FINAL REPORT

STUDY OF

IN SITU VOLATILIZATION OF SELENIUM
II. EVAPORATION PONDS

September 1989

Prepared under Contract
for the Federal–State
San Joaquin Valley Drainage Program

This report presents the results of a study conducted for the Federal-State Interagency San Joaquin Valley Drainage Program. The purpose of the report is to provide the Drainage Program agencies with information for consideration in developing alternatives for agricultural drainage water management. Publication of any findings or recommendations in this report should not be construed as representing the concurrence of the Program agencies. Also, mention of trade names or commercial products does not constitute agency endorsement or recommendation.

The San Joaquin Valley Drainage Program was established in mid-1984 as a cooperative effort of the U.S. Bureau of Reclamation, U.S. Fish and Wildlife Service, U.S. Geological Survey, California Department of Fish and Game, and California Department of Water Resources. The purposes of the Program are to investigate the problems associated with the drainage of irrigated agricultural lands in the San Joaquin Valley and to formulate, evaluate, and recommend alternatives for the immediate and long-term management of those problems. Consistent with these purposes, Program objectives address the following key areas: (1) Public health, (2) surface- and ground-water resources, (3) agricultural productivity, and (4) fish and wildlife resources.

Inquiries concerning the San Joaquin Valley Drainage Program may be directed to:

San Joaquin Valley Drainage Program
2800 Cottage Way, Room W-2143
Sacramento, California 95825-1898

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Under
U.S. Bureau of Reclamation
Contract No. 7-FC-20-05110

By
W. T. Frankenberger, Jr. and E. T. Thompson-Eagle
Department of Soil and Environmental Sciences
University of California, Riverside
Riverside, CA 92521

September, 1989

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EXECUTIVE SUMMARY

High selenium levels in agricultural drainage water and soil are a major problem for California farmers, environmentalists and state and federal legislators. Selenium is also a widespread contaminant throughout the U.S. including Arizona, Colorado, Montana, Nevada, New Mexico, South Dakota, Utah and Wyoming. Wildlife aberrations and toxicities are becoming apparent and solutions are being sought to remediate this problem. The agricultural drainage water in areas of the San Joaquin Valley is extremely saline and high in selenium. This saline, alkaline water is disposed of into large evaporation ponds. These ponds range in size between a few acres to over 750 acres. Each grower must sacrifice roughly 10 to 15% of his land to an evaporation pond facility in order to continue farming in certain areas of the San Joaquin Valley. It is estimated that over 7 million tons of salts including selenium are accumulating yearly in the farmlands of the San Joaquin Valley through irrigation practices. According to a 1989 U.C. Salinity/Drainage Task Force panel (a group of experts who were asked by the state and federal governments to assess and conduct research on the farming problems in the Valley) there are few preventative methods available on selenium accumulation. Biological deselenification may be a novel but practical method to deal with the yearly increase in selenium concentrations in evaporation ponds.

Selenium levels in some agricultural drainage evaporation ponds within the San Joaquin Valley are continuing to increase over time.

Many sites have levels that are well above the 1987 California State Water Resources Control Board water quality recommendation of 5 ppb Se for drainage water suitable for discharge into rivers.

The technology of biological deselenification is still in its infancy. Over the last two years, our laboratory has focused on research that dramatically accelerates this naturally occurring transformation. This microbial reaction converts toxic selenium compounds present in the water into a non-hazardous, gaseous form, dimethylselenide (DMSe) which is liberated into the atmosphere. DMSe is non-hazardous to rats. The scientific terms for this process include: selenium "biomethylation", "alkylation", "transmethylation", volatilization", and "deselenification". Although the organisms responsible for biomethylation are microscopic, they are able to multiply relatively fast in the presence of nutrients and make a substantial contribution to water deselenification in a relatively short period of time.

One objective we wanted to assess at the beginning of this project was whether selenium biomethylation occurs in evaporation pond water. While we already had some experience with selenium methylation in soils, there was some skepticism among fellow scientists as to whether it would occur directly in pond water without the influences of the sediment. Despite the harsh, saline conditions found in evaporation pond water, it was quickly discovered that biomethylation did indeed occur, albeit at very low levels, in water collected from these ponds. We were able

to confirm this by capturing the end-product, DMSe, and analyzing it using gas chromatography and mass spectroscopy. Bacteria and fungi were isolated which are resistant to selenium concentrations between 1 and 100 ppm. One particular isolate, Alternaria alternata, was found to produce large quantities of DMSe. This fungus was particularly efficient at methylating the common forms of selenium found in the water, selenite and selenate, even at extremely high concentrations and was stimulated by the addition of cofactors (chemicals which facilitate biological reactions) such as methionine and methyl cobalamine.

No selenium alkylation occurred in sterilized pond water. This confirmed that the process is a biological rather than a chemical reaction. Treatments such as increasing the temperature to 35°C, adding a fungal inoculum as well as carbon amendments consisting of simple sugars, complex carbohydrates, and amino acids increased biomethylation slightly. Not until proteins were added did we uncover an exciting discovery. Albumen (a protein found in the white of egg), casein (a milk protein) and gluten (a wheat protein) dramatically increased DMSe evolution by pond water at all of the concentrations tested. After 43 days of incubation, albumen, casein and gluten (2 g carbon L^{-1}) caused a 23%, 41% and 10% selenium loss from the inventory, respectively.

Encouraged by the discovery that proteins dramatically stimulated volatilization, other organic amendments were tested including saccharides (mono-, poly- and acidic), alcohols, fats and oils. None of these compounds

were used by the aquatic microorganisms to fuel the methylation reaction. We therefore decided to further investigate the stimulatory components of the milk-protein, casein and characterize its effects on Se methylation in pond water. Casein was heat-treated, boiled with acid, incubated with enzymes, purified, dialyzed, separated into its building blocks (proteins, peptides, amino acids, nitrogen) and minerals were removed. It was concluded that the active ingredient of casein is likely to be a peptide mixture. Casein did not appear to increase selenium methylation through any concurrent changes influencing the physicochemical and chemical characteristics of the water such as altered acidity/alkalinity, mineral content or chelation (binding) of pond water salts.

Our primary objective was to find sources of economical, readily available and unwanted protein/peptide sources that could be used in a water treatment process. All of the following by-products were good sources of proteins and stimulatory to the deselenification process: cottonseed and soybean meals (agricultural industry), cheese whey, whey protein (dairy industry by-products) and yeast sludge (food and beverage, e.g., beer industry).

Investigations with seleniferous pond water using microscopy techniques, antibiotic treatments, and microbiological counting techniques indicated that bacteria are the principal selenium methylating organisms in evaporation pond water. The addition of casein (4 g L^{-1}) to pond water not only stimulated biomethylation 25-fold but also increased the number of bacteria within the water by 1000-fold. We therefore

believe that proteins such as casein are important because of their nutritional value to the bacteria. The bacterial populations present in the drainage water are well adapted to their environment and resistant to a number of toxic trace elements including arsenic, boron, chromium, lead, molybdenum, selenium, silver and uranium naturally present in the pond water.

Optimum conditions for the protein-fuelled reaction were observed in a well-mixed, aerobic system. Growth attachment sites in the form of sand and glass beads were unnecessary for the successful multiplication and growth of the bacteria. The addition of trace amounts of reaction cofactors (methyl donors and reducing agents) further enhanced the methylation reaction in the water. The two major inorganic aquatic water species, selenite and selenate, were methylated equally well in the presence of protein. We found that methylation increased at lower water selenium concentrations. Artificially spiking the water with higher concentrations of selenium was not inhibitory. Dilution of the pond water with deionized water did not increase biomethylation despite the decrease in selenium, implying that selenium concentration, bacterial numbers and nutrients are far more crucial limitations to volatilization than high salinity. Another potential limitation to the process was high concentrations of nitrates and nitrites but the inhibitory concentrations were in excess of the natural levels of nitrogenous compounds found in evaporation pond water. Further increases in sulfates, a salt already present in extremely high concentrations in pond water, had no effect.

Field experiments were conducted directly in the evaporation ponds. We found that a single casein amendment (0.2 g C L^{-1}) caused a 38% selenium loss from the inventory of a San Joaquin Valley evaporation pond in a 142-day period.

Future studies should focus on successfully developing a deselenification water treatment process using the information collected from our laboratory and field studies. Further research is needed to develop various engineering techniques that are compatible with the previously researched optimum variables and also to continue screening economical nutrient sources which are needed to fuel the reaction. While the emphasis should be on environmental engineering it would also be of interest to pursue the genetic implications of biomethylation. Further field work is needed using continuous flow systems. We conclude that the acceleration of deselenification in evaporation pond water through the use of economical, available protein sources is a promising and novel bioremediation technique. It may be possible to use an activated sludge system, trickling filter or rotating biological contactors which would have the potential to remove and detoxify agricultural as well as industrial wastewater contaminated with Se.

CHAPTER 1

PROTEIN-MEDIATED SELENIUM BIOMETHYLATION IN EVAPORATION POND WATER

SUMMARY

Selenium is a contaminant of agricultural drainage water in areas of the San Joaquin Valley, CA and the cause of major toxicity problems among the wildlife. The objective of this study was to stimulate selenium dissipation from pond water through biomethylation. Laboratory studies showed that the process of Se biomethylation is protein/peptide-limited rather than nitrogen-, amino acid- or carbon-limited. Crude casein and its components, ∂- and β-caseins and peptides (1 g carbon [C] L^{-1} pond water) were equally stimulatory producing a >50-fold enhancement in dimethylselenide (DMSe) yield. Dephosphorylation of casein resulted in a 57% reduction in Se volatilization. Dialysis studies indicated that the active ingredient of casein is likely to be a peptide or peptide mixture. Casein did not appear to increase Se methylation through any physicochemical characteristic such as a change in pH or chelation of pond water ions. Alternative protein sources including cottonseed meal, cheese whey and yeast sludge dramatically increased methylation (29-fold, 300-fold and 41-fold, respectively) over unamended samples. A single casein amendment (0.2 g C L^{-1}) to water columns in the field caused a

a 38% Se loss from the initial inventory of a San Joaquin Valley evaporation pond in 142 days. We conclude that protein-mediated water deselenification is a promising and novel bioremediation technique to permanently remove Se on site.

INTRODUCTION

The disposal of saline agricultural drainage water is a major problem for farmers in the San Joaquin Valley, California. Currently, the drainage water is collected by tile drains and pumped into evaporation ponds. Because of the buildup of selenium (Se) salts in these waters, there is concern for the wildlife which is extremely vulnerable to the toxic effects of Se (Ohlendorf, 1989). Selenium volatilization within these saline agricultural drainage waters results in the permanent removal of Se with the release of a volatile Se species, dimethylselenide (Thompson-Eagle et al., 1989; Thompson-Eagle and Frankenberger, 1990), into the atmosphere. Previous studies have shown that inhaled DMSe is nonhazardous to rats when exposed to extremely high concentrations of $\leq$8034 ppm (W. T. Frankenberger, Jr. and U. Karlson, Dissipation of Soil Selenium by Microbial Volatilization at Kesterson Reservoir, U.S. Department of the Interior, Bureau of Reclamation, December 1988). Potentially this bioremediation technique may have applications for the safe disposal of toxic drainage water in the Central Valley of California as well as for other problem areas contaminated with Se in the western U.S. including the lower Colorado River Basin (Ohlendorf, 1989).

Previous studies (Thompson-Eagle and Frankenberger, 1990) indicate that the type of carbon source applied to Se-contaminated evaporation pond water is crucial to the methylating performance of the water. Casein, a complex milk protein, dramatically enhances biomethylation. This is not the first instance in which proteins have been linked with Se metabolism. Experiments in the 1930s and 1940s indicated that increasing the dietary protein in the form of casein decreased symptoms of Se poisoning or selenosis in mice and rats (Gortner, 1940; Lewis et al., 1940; Smith, 1939). The protein diets caused increased growth and decreased mortality, anemia, effusions and liver damage. Rats fed crude diets containing milk or commercial protein feeds prior to the injection of a subacute dose of labelled selenate exhaled 2 to 3 times as much volatile Se as those fed a purified basal diet (Ganther, 1965).

It is unclear what role proteins may have in both mammal and microbial methylation. Early Se toxicity studies with casein failed to pinpoint the active component of the protein responsible for the therapeutic effects observed in Se-poisoned animals. However, media components such as peptone, yeast extract and casamino acids have a high affinity for various metal ions and can thus modify their toxicity to microorganisms (Ganther, 1974; Ewan, 1989; Wrench and Campbell, 1981). It is well known that Se is associated with specific proteins such as glutathione peroxidase within the cells and fluids of birds and mammals (Ganther, 1974; Ewan, 1989; Wrench and Campbell, 1981) as well as with organic particles in seawater (Michael Siu and Berman, 1989). Cutter (1982) and Cutter and Bruland (1984) suggested that dissolved selenides exist in freshwater systems as peptide-bound selenoamino acids. The

tendency for Se to associate with organic matter such as protein amendments increases the likelihood that microorganisms will remove and metabolize the organic-sorbed Se from water.

Proteins are poorly metabolized by most microorganisms because of their failure to absorb such large substances. Utilization of proteins depends on the microbe's ability to produce exocellular proteases and/or a capacity to absorb the degradation products. The products of protein hydrolysis mainly consist of peptides and amino acids. Peptides stimulate bacterial growth directly but they may also affect growth indirectly through the suppression of toxic components within the environment (Matthews and Payne, 1975). This factor may be especially relevant to a matrix loaded with toxic elements, i.e., a "super saturated saline solution".

Few field studies have been performed to monitor biomethylation of metals and metalloids from aquatic systems. Most Se volatilization studies have been carried out in the laboratory. Terrestrial biogenic fluxes for Se are often estimated with respect to sulfur data Ross, 1984). Field atmospheric measurements of Se have been confined to volcano aerosols (Mroz and Zoller, 1975; Suzuoki, 1964), soils and landfills (W. T. Frankenberger, Jr. and U. Karlson, Dissipation of Soil Selenium by Microbial Volatilization at Kesterson Reservoir, U.S. Department of the Interior, Bureau of Reclamation, December 1988; Zieve and Peterson, 1984a, 1986), and air vapor collected from various marine, freshwater and sewage treatment sites (Jiang et al., 1983; Mosher and Duce, 1981; Reamer, 1978). No atmospheric concentrations of Se have been monitored directly from agricultural drainage water.

The objectives of this study were to i) investigate the stimulatory properties of the milk protein, casein and identify its active ingredient(s) in promoting methylation, ii) characterize the physicochemical properties of casein in pond water, iii) identify alternative protein sources for deselenification, and iv) monitor *in situ* Se methylation from evaporation pond facilities in the San Joaquin Valley, California.

MATERIALS AND METHODS

Chemicals

Alanine, arginine, asparagine, aspartic acid, casamino acids, casein, casein purified powder, ∂-casein, β-casein, κ-casein, chitin, cystine, cysteine, dephosphorylated casein, gliadin (crude), glutamic acid, glutamine, glycine, glycerol, histidine, isoleucine, leucine, lysine, L-methionine, phenylalanine, phosphoserine, proline, serine, threonine, tryptophan, tyrosine, valine, yeast extract and zein were all obtained from Sigma (St. Louis, MO). Agar was obtained from Difco (Detroit, MI) and lactose and sodium phosphate monobasic from Mallinckrodt (Paris, KY). Peptone was obtained from Nutritional Biochemicals Corp. (Cleveland, OH) and calcium chloride, barium chloride, potassium hydroxide, and ethylenediamine-tetracetic acid, disodium salt (EDTA) from Fisher Scientific (Fairlawn, NJ). Tropaeolin-O was obtained from ICN Biomedicals Inc. (Costa Mesa, CA). Cottonseed meal, safflower seed meal and soybean meal were obtained from Coast Grain Co. (Ontario, CA) and barley straw from the Citrus Experimental Station, Riverside, CA.

Sample collection and analysis

Water samples were collected from the Sumner Peck Ranch (Fresno County, California). The water samples were stored in Nalgene bottles, transported on blue ice in coolers and stored at 5°C. All samples were analyzed for pH, electrical conductivity (EC) and selenium (Se). Selenium was analyzed using atomic absorption spectrometry (AAS) with hydride generation. The instrument used was a Varian Spectra AA-10 atomic absorption spectrometer (Mulgrave, Victoria, Australia) with a VGA-76 vapor generator assembly. The operational conditions were as follows: acetylene, 2.4 mL min^{-1}; air, 6.3 mL min^{-1}; nitrogen, 90 mL min^{-1}; sample flow, 6.5 mL min^{-1}; conc. HCl flow, 1.2 mL min^{-1}; reagent flow, 0.6% $NaBH_4$/0.5% NaOH, 1.2 mL min^{-1}; lamp current, 10 mA; wavelength, 196.0 nm and slit width, 1.0 nm. All samples were treated with 0.2% $(NH_4)_2S_2O_8$, an equal quantity of concentrated HCl to give a sample acidity of 6 N and boiled for 1 h immediately before analysis. Filtration of the water samples was not necessary. Quality assurance was obtained by use of matrix spikes and matrix spike duplicates. Accuracy was maintained at 80-120% recovery, precision was within 15% relative standard deviation and the detection limit was 2 μg L^{-1}.

Mesocosms

Water samples were shaken vigorously and 75-mL aliquots were distributed aseptically into sterile 125-mL screw-capped Erlenmeyer flasks. The flasks were then capped with 70% ethanol-disinfected Mininert valves (Dynatech, Baton Rouge, LA) and each was placed on an orbital shaker (120 rpm) at room temperature (22°C ± 3). The headspace above the water

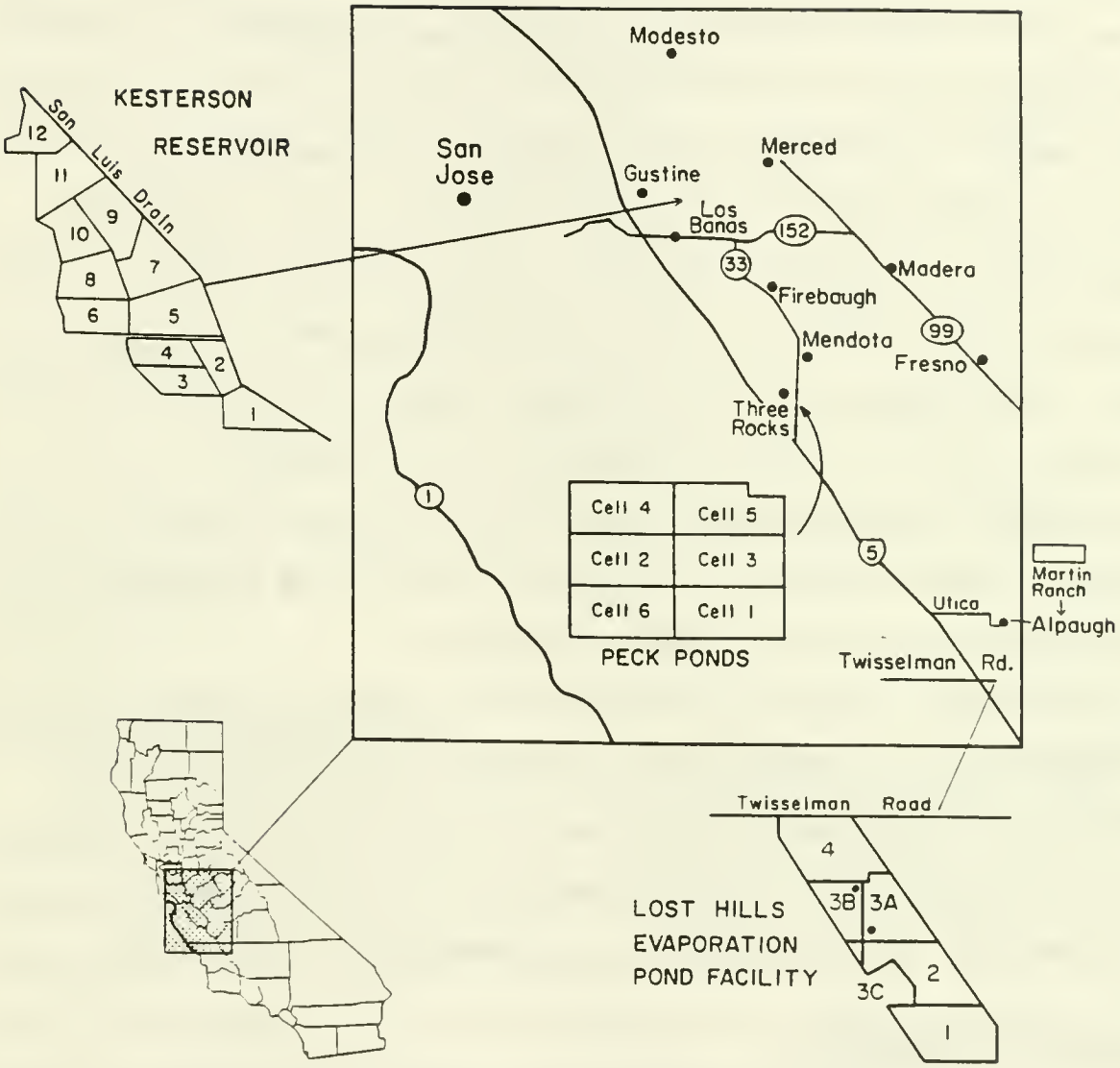

Fig. 1-1. Location of evaporation ponds in the Lost Hills District, the Sumner Ranch, the Martin Ranch and Kesterson Reservoir.

was analyzed for DMSe, the principal product of aquatic Se methylation, every 2 to 4 days using gas chromatography (GC). A 1-mL gas sample was withdrawn from each flask with a gas-tight Series 2 pressure-lok gas syringe (Alltech, Deerfield, IL) and injected directly into a Hewlett-Packard (Avondale, PA) Model 5890 GC, connected to a Hewlett-Packard 3393 integrator. The operational conditions were as follows: stainless steel column (10 m x 2.2 mm I.D.), liquid phase, 10% Carbowax 1000, solid support, Chrom-W-AW, 0.18-0.24 mm particle size (mesh 60/80); flame ionization detector; column temperature, 58°C rising to 80°C; injector temperature, 105°C; detector temperature, 125°C; carrier gas (He), 30 mL min^{-1}; air, 400 mL min^{-1}; H_2, 33 mL min^{-1}. After sampling, the headspace was evacuated for 20 min using a vacuum system under aseptic conditions (flow rate, 8 L min^{-1}). The flasks were then returned to the shaker to continue incubation.

Each treatment consisted of five replicates. Incubation periods of each experiment are specified in their corresponding tables and figures. Evolution of Se is expressed as DMSe released (µg Se L^{-1} pond water). Error bars in each figure depict the cumulative standard error of the mean. The data were statistically analyzed using one-way analysis of variance.

Casein-related experiments:

Casein purification, hydrolysis, denaturation and protein separation

Various casein components were analyzed for their total carbon (C) content using a modified Coleman nitrogen analyzer (Maywood, IL). Five mg of the sample were placed in an aluminum sample boat and covered with fines of a Cuprox catalyst before insertion into a quartz tube. The oxy-gen flow rate was 50 to 100 mL min^{-1} with a combustion temperature of

approximately 800°C. The CO_2 gas emitted from the sample was trapped in an absorption tower packed with glass beads containing 10.0 mL of 0.2 N KOH with Tropaoline-O. At the end of the combustion cycle, 5 mL of $BaCl_2$ were added plus 4 drops of phenolphthalein and the solution was titrated to the phenolphthalein end point with standardized 0.2 N HCl.

Agricultural drainage water (2.7 mg Se L^{-1}) in laboratory mesocosms was treated with one of the following casein compounds at a rate of 1 g C L^{-1} pond water: casein powder (48.8% C); purified casein powder (48.6% C); autoclaved casein powder (121°C, 115 lbs pressure for 15 min); autoclaved, purified casein powder; dephosphorylated casein powder (45.2% C); casamino acids (30.63% C); ∂-casein (41.2% C); β-casein (46.8% C); κ-casein (38.1% C); phosphoserine (17% C); and lactose (40% C).

Amino acid components of casein

Casein was analyzed for its amino acid content by the Biotechnology Instrumentation Facility, University of California, Riverside, using precolumn phenylthiodantoin derivatization and reverse-phase separation with an Applied Biosystems 420 derivatizer (Foster City, CA). On the basis of this analysis, equivalent molarities of the amino acids were added to deionized water (Table 1). The amino acid cocktail was filter-sterilized (0.22 µM, Gelman, Ann Arbor, MI) and added to pond water (2.1 mg Se L^{-1}) at an equivalent rate to 2 g C L^{-1} casein.

Dialyzable components of casein

Pond water (2.3 mg Se L^{-1}) was amended with 1 g C L^{-1} of either crude casein or casamino acids either as a free powder or within dialysis tubing (Gelman, molecular weight cutoff = 6,000 to 8,000).

Table 1. The amino acid composition of casein.

Amino acid residues	Composition of casein (2 g C L^{-1})	Composition of synthetic casein (2 g C L^{-1}) added to pond water
	(μM)	(μM)
Asparagine + aspartic acid	21.5	40
Glutamic acid + glutamine	35.3	40
Serine	9.6	10
Glycine	4.8	5
Histidine	10.4	10
Arginine	5.6	5
Threonine	5.6	5
Alanine	5.6	5
Proline	15.2	10
Tyrosine	5.6	5
Valine	8.8	5
Methionine	2.3	5
Cysteine + cystine	0	5
Isoleucine	5.6	5
Leucine	12.8	10
Phenylalanine	4.8	5
Lysine	11.9	10
Tryptophan	--	10

pH

Unamended and casein-amended (2 g C L^{-1} casein) pond water (2.5 mg Se L^{-1} pond water) were titrated with HCl or NaOH solutions and adjusted to pH 4, 5, 6, 7, 8, 9 and 10, respectively.

Nitrogen addition

Ammonium chloride was added at the rate of 0, 0.01, 0.1, 1, 10, 100 and 1,000 mM to pond water (2.13 mg Se L^{-1}).

Phosphate addition

Autoclaved sodium phosphate (monobasic) was added at concentrations of 0, 0.01, 0.1, 1, 10, 100 and 1,000 mM to mesocosms containing either unamended or casein-amended (2 g C L^{-1}) pond water (2.0 mg Se L^{-1} pond water).

Calcium addition

Autoclaved calcium chloride was added at concentrations of 0, 0.01, 0.1, 1, 10, 100 and 1,000 mM to mesocosms containing either unamended or casein-amended (2 g C L^{-1}) pond water (2.5 mg Se L^{-1} pond water).

Chelation

Autoclaved EDTA was added at concentrations of 0, 0.01, 0.1, 1, 10, 100 and 500 mM to mesocosms containing either unamended or casein-amended (2 g C L^{-1}) pond water (2.7 mg Se L^{-1} pond water).

Alternative protein sources

The following protein/peptide sources were added at a rate of 2.0 g L^{-1} to mesocosms containing pond water (2.1 mg Se L^{-1}): casein, gliadin, peptone, yeast and zein. Milk was added at a rate of 5 mL L^{-1} pond water.

Vegetative protein sources

The following vegetative protein sources were added at a rate of 4.0 g L^{-1} to mesocosms containing pond water (2.0 mg Se L^{-1}): cottonseed meal, safflower seed meal, soybean meal and wheat straw.

Dairy by-products

The following dairy by-products were added at a rate of 1 g protein L^{-1} to mesocosms containing pond water (1.8 mg Se L^{-1}): desalinized whey concentrate (2.5%), liquid whey protein concentrate (25%), whey protein concentrate powder (75% protein), yeast (6%), an evaporator concentrate produced from the permeate of ultrafiltration subjected to reverse osmosis, fermentation, alcohol removal and evaporation (4.25% protein) and an animal feed complex mixture consisting of evaporator concentrate and yeast (6.5% protein). Whey butter (0% protein) was added at 2 g L^{-1} pond water and unprocessed cheese whey (0.3% protein) at 0.25 g protein L^{-1} pond water.

Field measurements

Water columns consisting of Pyrex glass tubes (5.08 cm x 1.83 m) were submerged in water of an evaporation pond at the Sumner Peck Ranch facility and firmly pushed 14 cm into the sediment. The tubes were then capped with Teflon-sprayed (Fluoroglide, Norton, Wayne, NJ) rubber stoppers. Each stopper had a hole drilled in the center to accommodate an activated charcoal cartridge trap as designed by Karlson and Frankenberger (1988a) to collect the volatile Se evolved from the water. The water within the tubes was either unamended or amended with casein at 0.2 g C L^{-1} pond water. The charcoal cartridges were changed every 2-3 weeks and analyzed for Se by AAS. For comparison, two

parallel sets of water columns were obtained from the field and transported to the laboratory containing an air:water:sediment profile of the pond. One set was capped with rubber stoppers containing the charcoal traps and the second set with mininert valves (125-mL screw-capped Erlenmeyer flask threads were fused onto the head of the columns). Selenium trapped in the charcoal cartridges was eluted with 12 mL methanol into stirred concentrated nitric acid and the volume made up to 25 mL which deionized water. The sample was boiled for 6 hours until no further yellow fumes were present. Water was added to make up for the volume lost and the sample was boiled with an equal volume of concentrated HCl. The sample was then analyzed for Se by AAS as described previously. Columns capped with mininert valves and incubated in the laboratory were analyzed using GC as described previously. These columns were incubated for 142 days.

RESULTS

Casein-related experiments:

Casein purification, hydrolysis, denaturation and protein separation

Treatment of evaporation pond water with crude, autoclaved or hydrolyzed casein had comparable effects on Se biomethylation (Table 2). In contrast, both purification and dephosphorylation of the crude casein reduced its efficacy, the latter treatment producing a significant 57% decrease in DMSe production compared with crude casein. Phosphoserine produced 3% and lactose 1% of the DMSe emitted by crude casein-amended water. The protein components of casein stimulated Se methylation in the following order: β-casein > crude casein > ∂-casein >

κ-casein (Table 2) with little difference between the ∂- and β-casein treatments and the crude protein. Casamino acids (acid-hydrolyzed casein) were as active in increasing Se methylation as the crude casein compound itself (Table 2, Fig. 1-2).

Amino acid components of casein

A pure mixture of the constituent amino acids of casein only produced <5% of the biomethylation activity of crude casein, a significant decrease in DMSe emission (Table 2).

Dialyzable components of casein

Enclosing casein or casamino acids within dialysis tubing had only a slight effect on DMSe yields (casein powder, 120.2 µg Se L^{-1}; casamino acids powder, 104.0 µg Se L^{-1}; dialyzed casein, 104.5 µg Se L^{-1}; dialyzed casamino acids, 96.7 µg Se L^{-1}). There were no significant differences among the four treatments.

pH

The pH of the pond water had little effect on biomethylation except at pH 4. Water titrated to pH 4 caused a significant reduction (54.8 µg Se released L^{-1} pond water) in proteinmediated biomethylation compared with pH 8 water (212.6 µg Se released L^{-1} pond water, a 74% decrease). Unamended water at pH 10 produced 4-fold more DMSe than at pH 8 (pH 8, 3.7 µg Se L^{-1}; pH 10, 15.2 µg Se L^{-1}). Autoclaved pond water experiments indicated that the stimulation of methylation in unamended pond water under alkaline conditions was not caused by an abiotic reaction (data not shown). There was no difference in DMSe release from autoclaved pond water at pH 6, 9, or 10.

Table 2. Influence of casein and products of protein purification, separation, hydrolysis, dephosphorylation and denaturation on DMSe production by aquatic microflora.

Casein treatment (17 days incubation)	Active ingredient	DMSe ($\mu g\ Se\ L^{-1}$)[a]
Unamended	--	1.4 ± 0.2a [b]
Casein	crude casein	285.8 ± 58.0cd
121°C, 20 min	autoclaved, crude casein	252.1 ± 48.1bcd
Purification	pure casein	188.2 ± 27.2bcd
121°C, 20 min	autoclaved, pure casein	152.7 ± 21.3bc
Hydrolysis	casamino acids	234.4 ± 26.4bcd
Dephosphorylation	dephosphorylated casein	122.8 ± 13.3b
Phosphate separation	phosphoserine	9.17± 0.9a
Saccharide separation	lactose	3.06± 0.3a
Protein separation	∂-casein	226.6 ± 36.0bcd
	β-casein	313.1 ± 73.1d
	κ-casein	176.4 ± 39.3bc
Unamended	--	2.3 ± 0.9a
Amino acid separation	amino acid mixture	6.7 ± 1.7a

[a]Mean values are given with standard errors (n=5)

[b]Values with the same letters are not significantly different ($P < 0.05$)

Nitrogen addition

The addition of different concentrations of nitrogen in the form of ammonium chloride did not cause any increase in Se biomethylation over that of unamended pond water (Table 3).

Phosphate addition

Sodium phosphate did not increase biomethylation in the unamended pond water and in protein-amended pond water. Concentrations in excess of 10 mM reduced protein-mediated biomethylation 90% and 99% at 100 and 1,000 mM, respectively (Table 3).

Calcium addition

The addition of calcium chloride to the unamended pond water significantly increased Se methylation 2- and 8-fold, respectively, at the highest concentrations tested, 100 and 1,000 mM (Table 3) but only 1,000 mM $CaCl_2$ increased DMSe emission (2-fold) in autoclaved water (data not shown). In the presence of casein, 1,000 mM Ca significantly decreased DMSe yield by 86%.

Chelation

Chelation of pond water with EDTA increased biomethylation 2.7-fold at 0.01 mM but at concentrations above 0.1 mM, inhibition (>70%) was observed in unamended pond water. In the presence of casein, there was a 99% reduction in DMSe emission at concentrations of 10 mM EDTA and above (Table 3).

Alternative protein sources

Casein, gliadin and zein, caused a 47-, 77- and 25-fold increase in DMSe evolution, respectively, compared with the unamended pond water

Table 3. Influence of the addition of ammonium, phosphate, calcium and the chelating agent, EDTA on unamended and protein-mediated biomethylation in evaporation pond water.

Treatment[a]	Concentration	Incubation	Mean DMSe emission (μg Se L^{-1})[b]	
			Unamended	Casein-amended
	(mM)	(days)		
Nitrogen	0	19	0.7 ± 0.1	-
	0.01		0.3 ± 0.1	-
	0.1		0.5 ± 0.1	-
	1		0.2 ± 0.1	-
	10		0.4 ± 0.2	-
	100		0.6 ± 0.2	-
	1000		0.3 ± 0.2	-
LSD			0.6	
Phosphorus	0	17	1.2 ± 0.4	273.0 ± 44.3
	0.01		0.5 ± 0.2	341.4 ± 53.8
	0.1		1.2 ± 0.3	244.6 ± 53.8
	1		0.3 ± 0.1	290.8 ± 35.7
	10		0.5 ± 0.2	163.5 ± 12.8
	100		0.9 ± 0.4	27.8 ± 5.2
	1000		0.7 ± 0.2	1.3 ± 0.2
LSD			0.5	103.7

Table 3 (continued).

Treatment[a]	Concentration (mM)	Incubation (days)	Mean DMSe emission (µg Se L^{-1})[b] Unamended	Casein-amended
Calcium	0	17	2.5 ± 0.5	131.8 ± 25.9
	0.01		1.6 ± 0.1	124.1 ± 50.1
	0.1		2.9 ± 0.3	107.0 ± 11.9
	1		2.2 ± 0.2	70.7 ± 6.8
	10		2.9 ± 0.5	81.5 ± 6.6
	100		5.2 ± 1.3	111.9 ± 27.3
	1000		21.3 ± 1.3	18.4 ± 1.8
LSD			2.6	52.0
EDTA	0	16	2.5 ± 0.9	270.6 ± 59.4
	0.01		6.8 ± 1.9	328.3 ± 45.5
	0.1		0.2 ± 0.1	229.2 ± 47.5
	1		0.7 ± 0.3	259.8 ± 68.1
	10		0.4 ± 0.3	0.6 ± 0.3
	100		0.4 ± 0.2	0.6 ± 0.3
	1000		0.0	0.1 ± 0.1
LSD			0.9	66.8

[a]Treatments were as follows: nitrogen, ammonium chloride; phosphorus, sodium phosphate; calcium, calcium chloride and EDTA, ethylenediamine-tetracetic acid, sodium salt.

[b]Mean values are given with standard errors (n=5, LSD, $P < 0.05$).

(Fig. 1-2). The addition of peptone, milk and yeast caused a 60-, 41- and 41-fold stimulation in Se methylation, respectively.

Vegetative protein sources

The vegetative protein sources, cottonseed meal, safflower meal, soybean meal and straw caused a 29-, 13-, 18-, and 2-fold increase in DMSe evolution, respectively, compared with the unamended water (Fig. 1-3).

Dairy by-products

All of the dairy by-products tested were stimulatory to Se biomethylation in the following order: 75% whey protein (327-fold) > 25% liquid whey protein (188-fold) > yeast (111-fold) $\geq$ animal feed (98-fold) > cheese whey (74-fold) $\geq$ evaporator concentrate (70-fold) $\geq$ desalted whey (70-fold) > whey butter (4-fold) > unamended water (Fig. 1-4).

Field measurements

The headspace measurements by GC and charcoal traps gave similar results for volatile Se emissions from the laboratory-incubated water columns. Gaseous Se emissions were as follows: unamended (GC) 6.0 µg Se L^{-1}; unamended (charcoal) 4.5 µg Se L^{-1}; casein-amended (GC) 107.4 µg Se L^{-1}; casein-amended (charcoal) 127.2 µg Se L^{-1}. The addition of casein to the water columns not only significantly increased volatile Se emissions in the laboratory, but also in the field (Fig. 1-5). After 142 days, the casein-amended pond water evolved 22- and 28-fold more Se than the unamended water in the field and laboratory, respectively. Greater methylation occurred under field conditions compared with the laboratory. Unamended and casein-amended water columns produced 2.7and 2.1-fold more volatile Se, respectively, than when incubated in the laboratory.

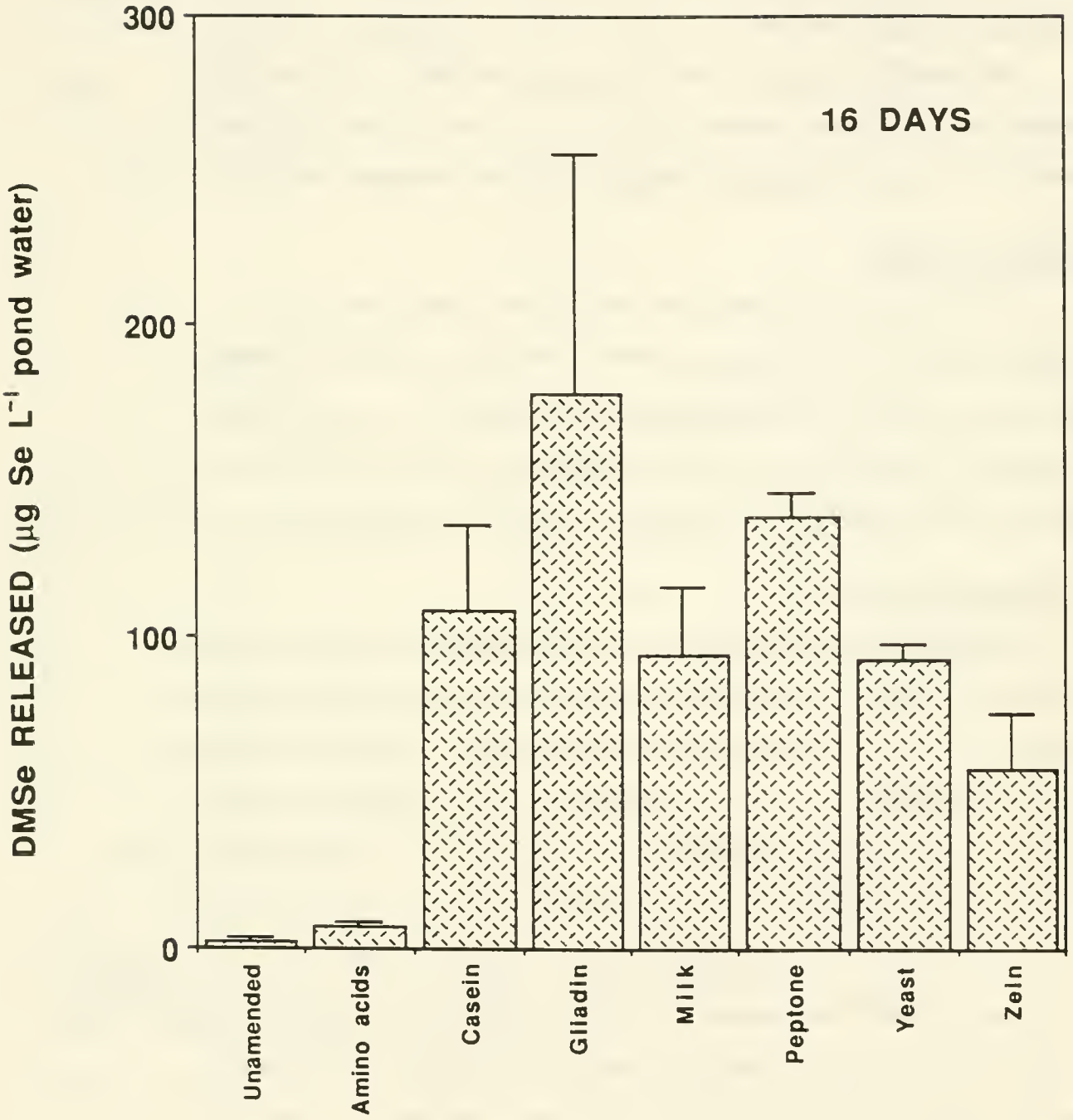

Fig. 1-2. Stimulation of Se biomethylation by protein sources and protein constituents.

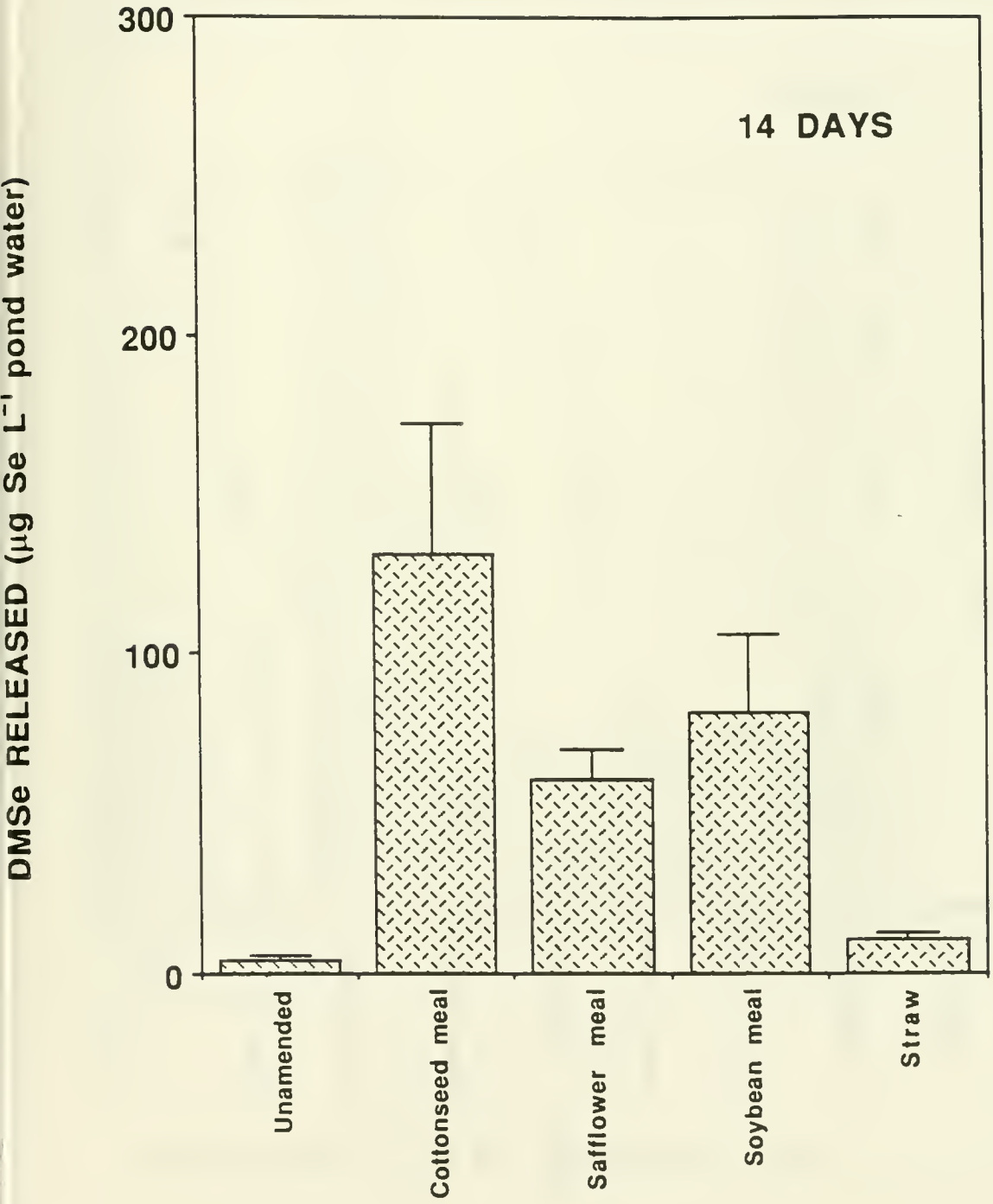

Fig. 1-3. Stimulation of Se biomethylation by vegetative protein sources.

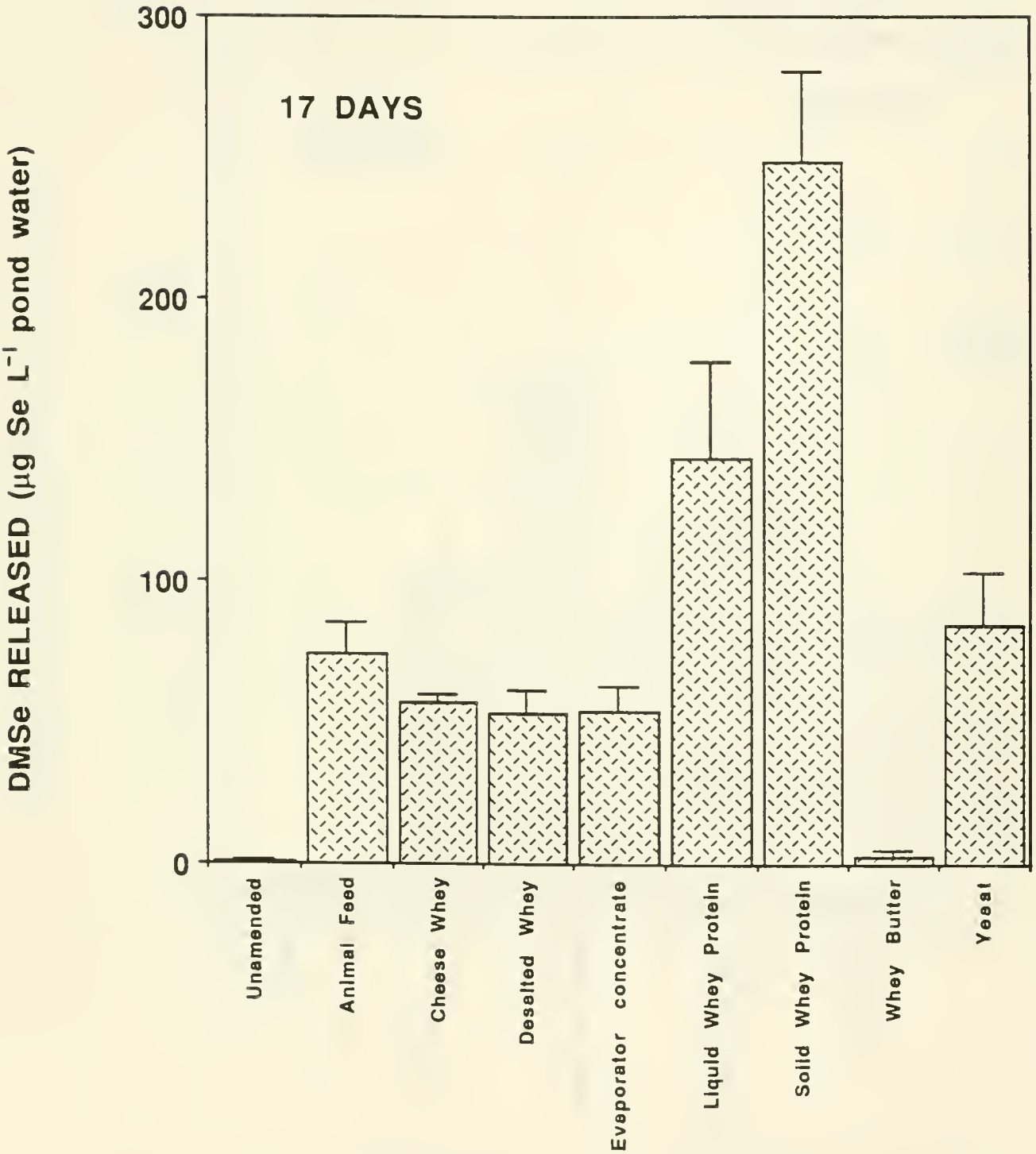

Fig. 1-4. Stimulation of Se biomethylation by dairy manufacturing by-products.

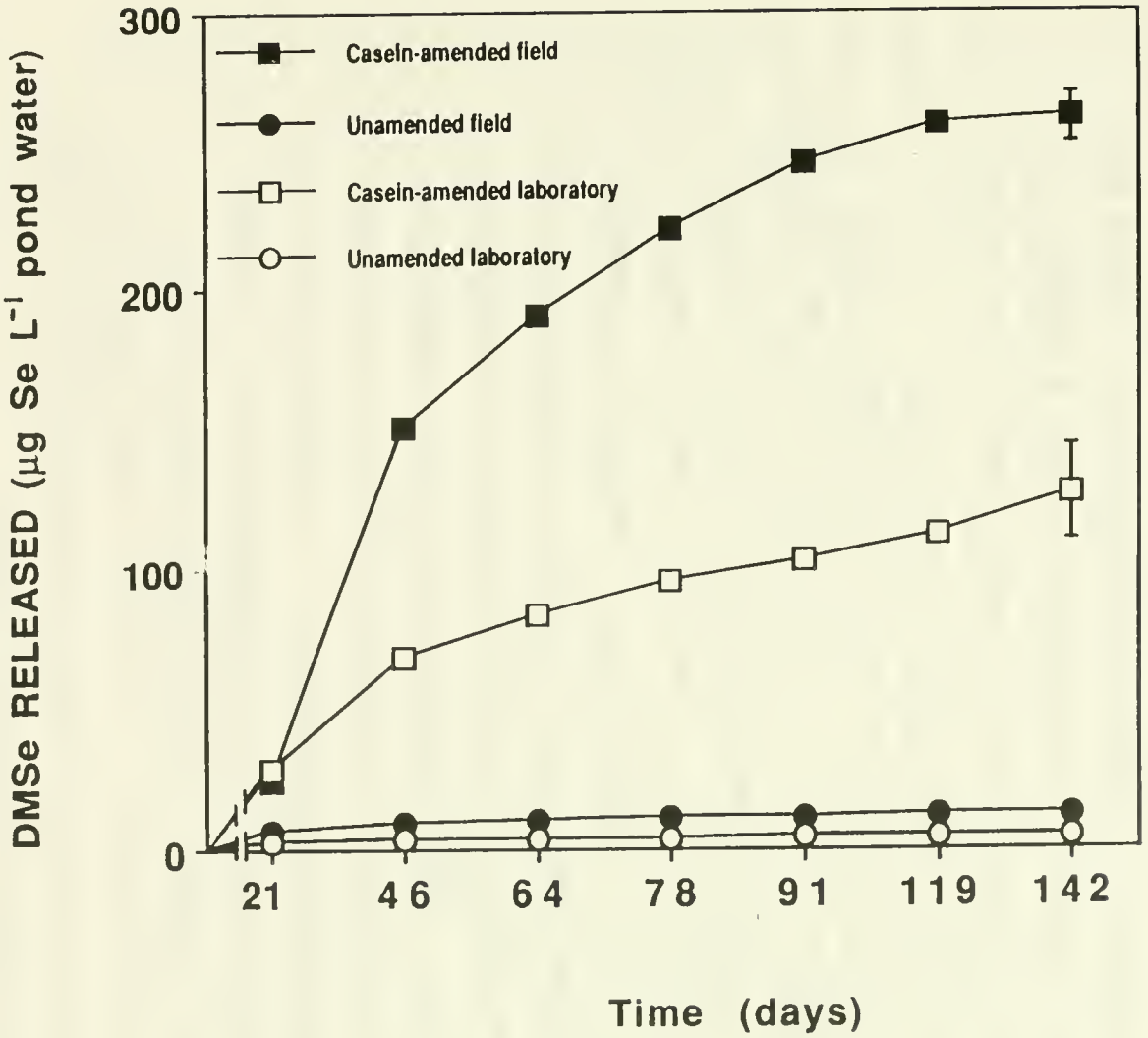

Fig. 1-5. Stimulation of Se biomethylation as a result of a single casein amendment (0.2 g C L^{-1}) in water columns incubated in the laboratory and the field.

DISCUSSION

Casein is a complex protein which may be further fractionated into the following components: ∂-caseins (48% of all casein protein), β-caseins (30 to 36%) and κ-caseins (13%) (Farrell and Thompson, 1988). Other constituents of casein include unincorporated peptides, amino acids, ammonium, carbohydrates, minerals, various vitamins and co-factors. The addition of casein to pond water not only increased the concentrations of available nutrients but also caused a change in the physicochemical characteristics of the water. Some of these changes may be beneficial to the microflora capable of methylating Se.

While all three protein components and various casein derivatives (purified, denatured, and acid- and enzyme-hydrolyzed casein) stimulated biomethylation, pure amino acids and inorganic nitrogen additions had little effect on DMSe emission. These findings indicate that the active ingredient is an organic nitrogenous compound with a level of organization greater than that of a collection of unincorporated amino acids. Dialysis experiments revealed that the active ingredient may be a peptide(s) since it bears a molecular weight of less than 6,000 to 8,000. Similar observations were noted by Pittman and Bryant (1964) with a rumen anaerobe, Bacteroides ruminocola. This organism could utilize peptide nitrogen efficiently in place of ammonium but not free amino acid-nitrogen. The length of the peptide chain was critical for utilization by the bacterium. In a nutritional study, Woolley (1946) found that mice grew slowly on amino acid-nitrogen, but growth increased greatly with the addition of casein to the diet.

Interestingly, there was relatively little difference between the amount of volatile Se detected as a result of dialyzed casein vs. casamino acids despite their differences in levels of organization. The protein subunits of casein are much larger than the pores of the dialysis tubing tested (∂-caseins 27,000 daltons; β-caseins 24,000 to 25,000 daltons; ∂-caseins, 21,000 daltons; [31]) and therefore were not available for microbial uptake. However, the dialysis tubing would allow the unincorporated peptide components of the casein to leak through. Many bacteria possess the ability to secrete extracellular proteases and peptidases which can breakdown proteins and peptides into smaller units suitable for bacterial uptake (Brown, 1982). While proteases tend to have relatively low molecular weights, e.g., pepsin (34,500), trypsin (24,000), lysozyme (14,400) and purified protease from Streptomyces caespitosus (15,000) (7,19, Sigma technical information on protease [Product P-0384], Sigma Chemical Co., 1989 Biochemicals, organic compounds for research and diagnostic reagents catalog technical service phone line), the bacterial proteases are still too large (>8,000) to pass through the dialysis tubing used in these experiments. Dixon and Webb (1979) reported that the cut-off point for catalytic activity is usually about 10,000 daltons. It is more likely that the indigenous microflora within the casein itself decomposed the protein inside the dialysis tubing upon submergence, allowing the protein digests to diffuse out into the medium.

Crude casein has some unique physicochemical characteristics. It is possible that as well as providing a source of nutrients to the aquatic microflora, it may also alter the physicochemical characteristics of the pond water which would in some way favor microbial methylation. Casein decreases the pH of evaporation pond water and is a strong adsorber of divalent cations. It can also provide a physical niche for microbial colonization increasing the BOD of the water. However, pH had relatively little effect on protein-mediated Se methylation except for inhibition at pH 4. This is in contrast to the findings of Fagerstrom and Jernelov (1972) who found that the pH optimum for mercury methylation is 4.5. However, low pH decreases the solubility and biological availability of Se in soil and aquatic systems (Weres et al., 1989), which would also decrease its availability for biomethylation. Inactivation of methylating bacteria may have occurred at this acidic pH range. It was noted that in unamended pond water titrated to pH > 9, salt crystallization visibly occurred and methylation increased 3-fold. This phenomenon was not observed in protein-amended water perhaps because the salts may have already complexed with the protein regardless of pH. Evaporation pond water is very high in salts (electrical conductivity is generally >20 dS m^{-1}). The addition of sodium hydroxide may have caused some of the more inhibitory salts to be removed from solution. However, selenium would still be available for methylation because of its high solubility. The increased pH may have made Se more available to the bacteria, or decreased competition

from other non-methylating competitors. Studies with sterile water have indicated (data not shown) that the increase in DMSe production at pH >9 was not solely the result of a chemical reaction.

Casein contains the elements calcium and phosphorus. The addition of sodium phosphate or phosphoserine, a major source of phosphate ions in casein, to unamended pond water had very little effect on Se methylation. Cox and Alexander (1974) found that concentrations of up to 7.35 mM phosphorus in the form of KH_2PO_4 had no effect on DMSe production by Candida humicola in the presence of 1,000 µg mL^{-1} selenate. In contrast, the synthesis of trimethylarsine by the same fungus from arsenate, arsenite and monomethylarsonate (but not from dimethylarsinate) was inhibited by phosphate (Cox and Alexander, 1974). In this study, protein-mediated Se methylation was inhibited by concentrations above 10 mM KH_2PO_4. The salt crystallization phenomenon observed at high pH values also occurred when extremely high concentrations of calcium (100 and 1,000 mM) were added to the pond water.

Selenium biomethylation was also demonstrated in the field. Unamended pond water and sediments evolved alkylselenides at extremely low rates (0.1 µg Se L^{-1} d^{-1}). Measurable concentrations of atmospheric alkylselenides (including DMSe) have been detected by various workers above areas relatively low in Se. Levels of up to 2.4 ng m^{-3} were detected above a lake (Jiang et al., 1983), up to 0.4 ng m^{-3} in coastal regions (Mosher and Duce, 1981) and 0.2 to 5.4 ng m^{-1} near a sewage treatment plant (Reamer, 1978). While methylation of Se in unamended evaporation pond water was extremely low, casein-amended water increased volatilization over 20-fold with an emission rate of 2.2 µg Se L^{-1} d^{-1}.

This indicates that Se biomethylation can be dramatically enhanced *in situ* under prevailing evaporation pond conditions. The amount of DMSe evolved from water columns in the field was significantly greater than that produced in the laboratory. This information indicates that previous laboratory experiments may have underestimated the efficiency of Se biomethylation in agricultural drainage water.

The findings that various proteins and protein-containing compounds are stimulatory to Se removal may be useful for the detoxification of seleniferous waters. A particularly promising source of proteins is the dairy industry which generates a variety of by-products including large quantities of whey proteins and yeasts. For many of these products, disposal is a problem. These compounds need to be assessed in continuous flow bioreactor studies to test their effectiveness and economic viability in the deselenification of agricultural drainage water.

CONCLUSION

Proteins dramatically stimulate Se biomethylation. Casein does not appear to increase Se methylation in pond water through any physicochemical characteristic such as a change in pH or chelation of pond water ions. Among the casein purification and denaturation treatments, only dephosphorylation had any significant effect on biomethylation. The active ingredients of this protein appears to be a mixture of simple peptides with a molecular weight of less than 6,000 to

8,000. The process of Se biomethylation is protein/peptide-limited rather than ammonium-, amino acid- or carbon-limited. These studies show that biomethylation is not casein-specific but stimulated by a wide variety of protein-containing compounds. Unwanted dairy by-products may be a valuable resource of proteins for deselenification. Protein-enhanced Se biomethylation was more effective under field conditions than in the laboratory. Further studies are needed to determine whether protein-mediated biomethylation can be optimized through the addition of coenzymes, methyl donors and aeration as well as the addition of specific microbial inoculants. Continuous flow pilot studies in the field are needed to test this promising detoxification technique.

CHAPTER 2

MICROBIAL TRANSMETHYLATION OF SELENIUM IN ALKALINE, SALINE POND WATER

SUMMARY

Selenium (Se) transmethylation is carried out by a consortium of aquatic bacteria in saline and alkaline ponds throughout the San Joaquin Valley, California. The methylated product, dimethylselenide, is a volatile gas that is 500-700 times less toxic to rats than aqueous selenite [Se(IV)] and selenate [Se(VI)]. The addition of casein (4 g L^{-1}), a milk protein, stimulated Se biomethylation 25-fold and increased bacterial numbers 1,000-fold. Optimum Se methylation by the bacterial consortium was observed in a well-mixed, aerobic system. The consortium was resistant to a number of broad spectrum bactericides and fungicides but not to 100 mg L^{-1} penicillin G or polymyxin B sulfate. The protein-mediated bacterial transformation was enhanced by the presence of trace amounts (10 μM) of homocysteine and reduced glutathione. Methylation of Se(IV) and Se(VI) species was equally efficient. Pond waters collected from different sites in the Central Valley contained 0.01, 0.23, 0.33, 0.98, 1.99 and 2.19 mg Se L^{-1} and as the Se inventory increased, the percentage of Se removal decreased from 100 to 20.9, 19.8, 6.3, 8.4 and 8.6%, respectively. Increasing the Se concentration from 2 to 4, 7, 12, 22 and 102 mg Se L^{-1} did not alter DMSe emission but the percentage of Se removal decreased from 8 to 3.8, 2, 1.3, 0.7 and 0.1%, respectively. Dilution of pond water salinity (70 to 20 dS m^{-1})

and Se concentration (2.1 to 0.4 µg Se L^{-1}) decreased biomethylation. Volatilization was inhibited by 0.1 and 1 M NO_3^- and NO_2^- but not SO_4^{2-}. Transmethylation of Se in a bioreactor containing aerated, well-mixed, protein- and coenzyme-amended agricultural drainage water could be a feasible method to reduce the Se inventory in the Central Valley.

INTRODUCTION

Microorganisms play a dominant role in modifying, activating and detoxifying organometallic compounds including toxic metals and metalloids in the environment (Saxena and Howard, 1977). Natural evolution of gaseous selenium (Se) from the lithosphere is an important link in the geochemical cycle of Se. It is estimated that approximately 50% of the Se within the atmosphere is derived from natural sources (Mosher and Duce, 1989) and 25-40% of this is in the gaseous form (Nriagu, 1989). The volatile products of Se transmethylation are dimethylselenide (DMSe) and dimethyldiselenide (DMDSe). The major alkylated species emitted from alkaline, seleniferous soils and water is DMSe (Karlson and Frankenberger, 1988b, 1989; Thompson-Eagle et al., 1989). Dimethylselenide is 500 to 700 times less toxic to rats than aqueous selenite [Se(IV)] and selenate [Se(VI)] (Franke and Moxon, 1936; McConnell and Portman, 1952). Microbial, macrofloral and macrofaunal Se methylating organisms have been reported in soils, sludge and various food sources (Doran, 1982) but little is known about Se methylation in the aquatic environment. Chau et al. (1976) have isolated Aeromonas sp., Flavobacterium sp. and Pseudomonas sp. from lake sediments active in methylating Se.

Both Se toxicity and Se requirements have been reported for various microorganisms. In general, Se is toxic to many organisms when present in high concentrations and resistance to this element by pathogens such as Salmonella spp. and Shigella spp. is often used as a diagnostic tool. The concentration appears to be critical since Se is an essential element for the growth of certain bacteria such as Escherichia coli, Clostridium thermoaceticum and Clostridium stricklandii, where it is a component in formate dehydrogenase and glycine reductase enzyme systems (Peterson et al., 1981).

Selenium is present in California agricultural drainage water due to the geology of the area and the high, saline water table. Animal toxicities have occurred as a result of Se poisoning (Ohlendorf, 1989). The drainage water from the saline soils is diverted into large ponds, where evaporation continuously concentrates the salts causing trace elements such as Se, arsenic, molybdenum and uranium to accumulate to toxic levels. While there are minor differences between the physicochemical characteristics of evaporation pond waters in different areas of the Central Valley, they share many similarities. The dominant ions in the seleniferous water are usually sodium, sulfate and chloride, each of which are present at concentrations well in excess of 1,000 mg L^{-1}. The water can be described as a super-saturated salt solution (alkaline, saline brine). The water levels in the evaporation ponds are fairly shallow, approximately one meter in depth and decrease during the hot summer months. Wading birds such as black neck stilts and American avocets can be seen feeding both on the shoreline and when the water levels decline sufficiently, in the water itself.

Studies on the biota of alkaline, saline environments have until recently been restricted to macroflora and macrofauna (Grant and Tindall, 1986) but it is well known that in alkaline environments, species diversity decreases as the salinity and alkalinity increases (Grant and Tindall, 1986; Reed, 1986). Microbial Se transmethylation reactions in alkaline, saline waters are likely to be of significance in the global cycling of Se. The objective of this study was to optimize the natural process of water deselenification through the stimulation of microbial alkalinophile growth and Se transmethylation in the presence of proteins. Factors that influence methylation of metals and metalloids such as Se are generally the same as those which affect other microbiological transformations (Saxena and Howard, 1977). Parameters which were examined for their ability to enhance biomethylating microorganisms included: i) aeration and mixing, ii) provision of suitable bacterial colonization surfaces, iii) Se concentration and species, and iv) the presence of enzyme activators, methyl donors and reducing agents. Inhibitory factors of the transmethylation reaction were also evaluated including the effects of: i) salinity, and ii) high concentrations of nitrates, nitrites and sulfates on deselenification. If volatilization of Se can be sufficiently optimized and potential inhibitory factors determined, it may be possible to design a bioreactor which would have the potential to remove and detoxify agricultural as well as industrial wastewater contaminated with Se.

MATERIALS AND METHODS

Reagents

Adenosine, S-adenosylmethionine, betaine, casein, casein purified powder, choline chloride, chlortetracycline, crystal violet, cycloheximide, dimethylglycine, formate, reduced glutathione, dL-homocysteine, L-methionine, methylcobalamine, nystatin, penicillin G, polymyxin B sulfate, sodium dichromate and streptomycin sulfate were all obtained from Sigma (St. Louis, MO). Agar (Bitek) and R_2A agar were obtained from Difco (Detroit, MI). Sodium nitrate and sodium nitrite were obtained from Mallinckrodt (Paris, KY), while sodium selenite and sodium selenate were obtained from Alfa Products (Denver, MA). Peptone was obtained from Nutritional Biochemicals Corp. (Cleveland, OH) and calcium chloride and methanol from Fisher Scientific (Fairlawn, NJ).

Water sampling

Water samples were collected from the Sumner Peck Ranch (Fresno County, CA), Lost Hills Westfarmers ponds (Kern County, CA) and the Martin Ranch (Tulare County, CA) (Fig. 1-1). The water samples were stored in nalgene bottles, transported on blue ice in coolers and stored at 5°C. All samples were analyzed for Se within 3 days of receipt by atomic absorption spectrometry (AAS) with hydride generation as described in Chapter 1.

Gas chromatography

The headspace above the pond water was analyzed for DMSe by withdrawing a 1-mL gas sample with a gas-tight Series 2 pressure-lok gas syringe (Alltech, Deerfield, IL) and injecting it directly into

a gas chromatograph (GC). The GC analysis was performed as described previously (Chapter 1).

Method of assay

Water samples were shaken vigorously and 75-mL aliquots were distributed aseptically into sterile 125-mL screw-capped Erlenmeyer flasks. The flasks were then capped with 70% ethanol-disinfected mininert valves (Dynatech, Baton Rouge, LA) and each was placed on an orbital shaker (120 rpm) at room temperature (22 $\pm$ 3°C). The headspace of each replicate was sampled once by GC every 2 to 4 days and after sampling the headspace was evacuated for 20 min using a vacuum system under aseptic conditions (flow rate, 8 L min^{-1}). The flasks were then returned to the shaker to continue incubation. Each treatment consisted of 5 replicates. Evolution of Se was expressed as DMSe released (µg Se L^{-1} pond water). Standard error bars of the cumulative mean are illustrated in all figures at the end of incubation.

Bacterial populations

Peck cell 3 water (2.2 mg Se L^{-1} pond water) was set up in laboratory mesocosms and treated with casein (4 g L^{-1} pond water) and shake-incubated (120 rpm) for 14 days at room temperature (22 $\pm$ 3°C). The pond water of each mesocosm was serially diluted in sterile deionized water and 3 replicate aliquots of each dilution were then plated out onto R_2A medium according to the method of Miles and Misra (1933). The medium was made up with either 10 mg L^{-1} Se(IV) as sodium selenite or Se(VI) as sodium selenate. Colony-forming units (cfu) were enumerated.

Antimicrobial agents

Various antimicrobial agents were added to Peck cell 3 water (1.8 mg Se L^{-1} pond water) containing 2 g casein L^{-1} and incubated for 18 days. These agents were applied at the following concentrations: chlortetracycline, 100 µg mL^{-1}; crystal violet, 10 µg L^{-1}; cycloheximide, 200 µg mL^{-1}; nystatin, 200 µg mL^{-1}; penicillin G, 100 µg mL^{-1}; polymyxin B sulfate, 100 µg mL^{-1}; penicillin G/polymyxin B sulfate, 50/50 µg mL^{-1}; sodium dichromate, 50 µg mL^{-1} and streptomycin sulfate, 100 µg mL^{-1}. At the end of incubation, each treatment replicate was enumerated by direct counts using a Neubauer Hausser counting chamber (Hylite, Ultraplane). Microbes were enumerated in ten different squares (1/400 sq mm x 1/10 mm deep) per aliquot. The average number per replicate per mL were calculated from the dilution and aliquot size.

Coenzymes

The following coenzymes and methyl donors (10 µM) were added to mesocosms containing Peck cell 3 water (2.1 mg Se L^{-1} pond water) and incubated up to 21 days: adenosine, S-adenosylmethionine (SAM), betaine, choline chloride, dimethylglycine, formate, reduced glutathione, DL-homocysteine, L-methionine and methylcobalamine. Peptone at 20 mg L^{-1} pond water was also added to an identical set of mesocosms.

Relationship between total selenium concentration, species, EC, pH and biomethylation

Water was collected from 6 different evaporation ponds in the Central Valley: Peck cell 1, Peck cell 3, Peck cell 5, Martin Ranch, Lost Hills Westfarmers Pond 4 and Lost Hill Westfarmers Pond 5. Bio-

methylation rates were monitored in laboratory mesocosms containing 4 g peptone L^{-1} pond water. Peck cell 3 pond water (1.9 mg Se L^{-1} pond water) containing 4 g peptone L^{-1} was amended with either sodium selenite or sodium selenate at the following concentrations: 0, 2, 5, 10, 20 and 100 mg Se L^{-1} pond water. On completion of DMSe monitoring (17 days), an aliquot from each flask was streaked onto modified R_2A agar (amended with 2 g L^{-1} sodium chloride) and grouped on a scale of 1 to 5 according to the growth density of the microbial colonies.

Reduction in salinity and Se concentration

Pond water (2.1 mg Se L^{-1} pond water) was diluted with sterile deionized water to give the following dilutions: 0, 20%, 50%, 80% and 100% which corresponded to the following electrical conductivity (EC) values: 70, 60, 40, 21 and 0.03 dS m^{-1}. Casein was added at a rate of 4 g L^{-1} pond water and mesocosms were incubated for 16 days.

Aeration and agitation

Laboratory mesocosms containing Peck cell 3 water (2.9 mg Se L^{-1} pond water) were incubated under nitrogen or air, either stationary or on an orbital shaker (120 rpm) with or without casein (4 g L^{-1} pond water) for 16 days. Air was removed from the headspace of the nitrogen-atmosphere flasks by displacement with nitrogen gas for one hour before commencement of the experiment. After each sampling, the nitrogen-atmosphere flasks were flushed with nitrogen for 20 minutes instead of air.

Attached growth surfaces

The following materials were added to mesocosms containing Peck cell 3 water (1.6 mg Se L^{-1} pond water); a 10-cm length of ring lace

(Dodwell Industrial, Tokyo, U.S. supplier: R. Hurst, Portland, OR); 3 g of 3-mm glass beads (American Scientific Products, McGraw Park, IL) and 0.3 g of 0.45-0.52 mm glass beads (Thomas Scientific, Swedesboro, NJ). These materials and an additional polyurethane polymer treatment (30 parts per inch, Filtren TR, Reticel, Division of NV Gechem, Wetteren, Belgium) were also added to peptone-amended (1 g L^{-1}) Peck cell 3 water and were incubated for 16 days.

Nitrates and nitrites

Sodium nitrate and sodium nitrite were added at a concentration of 0, 0.1 and 1 M to Peck cell 3 water (1.8 mg Se L^{-1} pond water) with and without peptone (1 g L^{-1} pond water) and incubated for 18 days.

Sulfates

Sodium sulfate was added to Peck cell 3 water (2.1 mg Se L^{-1} pond water) at 0, 0.1 and 1 M, with and without peptone (1 g L^{-1} pond water) and incubated for 18 days.

Alcohols

Methanol was added to laboratory mesocosms containing Peck cell 3 water (1.8 mg Se L^{-1} pond water) at the following concentrations: 0, 0.25, 2.5 and 25 mL L^{-1} pond water and incubated for 14 days.

Oils

The following oils were added to Peck cell 3 water (2.9 mg Se L^{-1} pond water) at a rate of 2 g C L^{-1}: codliver oil (76.9% C), corn seed oil (77% C), glycerol (39% C), peanut oil (77% C) and sunflower oil (77% C) and incubated for 16 days.

RESULTS

Bacterial populations

The number of bacteria isolated on seleniferous R_2A agar increased 1,000-fold when evaporation pond water was amended with casein (Table 4). The selenium species, [Se(IV) vs. Se(VI)] had little effect on the number of bacteria isolated from the pond water.

Antimicrobial agents

The fungicidal agent, cycloheximide, stimulated Se biomethylation both initially and after 18 days (1.2-fold), while nystatin had no effect and sodium dichromate was stimulatory only after a period of one week, the final stimulation being 1.2-fold after 18 days (Table 5). All three fungicides caused a decline in total microbial numbers compared with the controls: cycloheximide, 61%; nystatin, 48% and sodium dichromate, a 59% decline. Biomethylation rates decreased initially in the presence of the Gram-positive bactericides, crystal violet (40%) and penicillin G (55%), and the Gram-negative bactericide, polymyxin B sulfate (48%). At the end of the experiment, inhibition of DMSe emission by crystal violet was 0, penicillin G, 47% and polymyxin B sulfate, 35%. Only crystal violet decreased the final microbial count causing a 57% decline in microbial numbers. The broad spectrum antibiotics tested, chlortetracycline and streptomycin sulfate, had very little effect on DMSe emission. Chlortetracycline decreased the microbial numbers by 62% while streptomycin sulfate caused a 1.58-fold increase in numbers. The morphology of the bacteria observed in casein-amended water included cocci, small and large rods, coryneforms, and helixes. The microorganisms present were unattached, paired, in chains and in clumps. Virtually no algae were

Table 4. Stimulation of bacterial populations and biomethylation through the addition of the milk protein, casein.

Pond water treatment	Incubation (days)	R_2A medium (cfu) Se(IV)	R_2A medium (cfu) Se(VI)	DMSe emission ($\mu g\ Se\ L^{-1}$)
Unamended	0	3.50×10^3	3.25×10^3	ND[a]
Unamended	18	2.80×10^3	4.00×10^3	5.9
Casein ($4\ g\ L^{-1}$)	18	5.15×10^6	4.45×10^6	149.4

[a]Not determined

Table 5. Influence of antimicrobial agents on selenium methylation in evaporation pond water.

Antibiotic	Conc.	Target micro-organism[a]	Average DMSe emission ($\mu g\ Se\ L^{-1}$ pond water)[b]
Control	0	0	166.8 ± 20.0
Crystal violet	10	B+	171.0 ± 13.5
Penicillin G	100	B+	89.0 ± 9.5
Polymyxin B sulfate	100	B-	108.9 ± 19.9
Chlortetracycline	100	B+,B-	150.6 ± 20.9
Streptomycin sulfate	100	B+,B-	149.7 ± 19.5
Penicillin G/ Polymyxin B sulfate	50/50	B+,B-	129.7 ± 30.8
Cycloheximide	200	F	197.7 ± 21.7
Nystatin	200	F	159.3 ± 17.6
Sodium dichromate	50	B-,F	198.7 ± 18.4
LSD			63.0

[a]Abbreviations: F, fungi; B^+, Gram-positive bacteria; B^-, Gram-negative bacteria.

[b]Mean values are given with standard errors (n=5, LSD, $P < 0.05$)

present except for an occasional diatom including Navicula sp. and no fungal filaments were observed. Some protozoans and invertebrates such as rotifers were observed grazing on the bacteria.

Coenzymes

Reduced glutathione, DL-homocysteine, and L-methionine promoted methylation of Se in unamended pond water 21-, 71-and 64-fold, respectively (Table 6). However, when the pond water was amended with peptone, L-methionine was no more stimulatory than the control while reduced glutathione and homocysteine increased methylation 5- and 14-fold, respectively. Glutathione and homocysteine but not methionine stimulated Se methylation even under sterile conditions. The abiotic production of DMSe by reduced glutathione and homocysteine after 3 days of incubation was 61% and 100%, respectively, of the values generated under non-sterile conditions and 53% and 26% of the DMSe produced biologically in 21 days. Adenosine, betaine and formate had no effect while dimethylglycine, methylcobalamine and SAM all inhibited methylation in the presence of peptone, causing a 71%, 37% and 40% decrease, respectively, in DMSe production.

Relationship between total selenium concentration, species, EC, pH and biomethylation

The evaporation pond waters tested for Se methylation capacity contained between 20 and 2190 µg Se L^{-1} (Table 7). The pH of these waters ranged between 8.00 and 9.17 and the EC between 11 and 68 dS m^{-1}. Although methylation was greater at higher concentrations of Se (pond

Table 6. Influence of coenzymes on DMSe production by aquatic microflora in evaporation pond water.

Coenzyme	Average DMSe emission[a] ($\mu g\ Se\ L^{-1}$)	
	Unamended	Peptone-amended
Unamended (15 d)	0.9 ± 0.2	3.4 ± 0.4
Adenosine	0.7 ± 0.1	3.9 ± 0.6
Betaine	0.7 ± 0.2	4.6 ± 0.8
Formate	0.9 ± 0.2	3.5 ± 0.2
LSD	0.5	2.5
Unamended (18 d)	0.1 ± 0.0	3.0 ± 1.0
S-adenosylmethionine	0	1.8 ± 0.5
Methionine	6.4 ± 1.8	3.8 ± 0.8
Methylcobalamine	0.1 ± 0.1	1.9 ± 0.3
LSD	3.1	2.4
Unamended (21 d)	0.3 ± 0.2	2.4 ± 0.4
Choline chloride	0.1 ± 0.1	0.2 ± 0.1
Dimethylglycine	0.5 ± 0.1	0.7 ± 0.1
Glutathione (reduced)	8.0 ± 0.7	11.0 ± 1.7
Homocysteine	21.4 ± 4.3	34.6 ± 2.1
LSD	6.5	2.8
Sterile water (3 d)	0	
Sterile betaine	0	
Sterile glutathione (reduced)	4.2 ± 0.3	
Sterile homocysteine	5.5 ± 0.4	
Sterile methionine	0.1	

[a]Mean values are given with standard errors (n=5, LSD, $P < 0.05$).

Table 7. Relationship between total Se, speciation, EC, pH, and biomethylation.

Water sample[a]	Collection date	pH	EC (dS m^{-1})	Total Se (mg L^{-1})	% Se loss after 17 d incubation	Micro-organisms[b]
Water MR-1	4/19/89	9.17	28	0.02	100	ND
Water W-3B	4/19/89	8.55	52	0.23	20.9	ND
Water W-3A	4/19/89	8.59	51	0.33	19.8	ND
Water P-1	4/19/89	8.00	11	0.98	6.3	ND
Water P-3A	4/19/89	8.67	58	1.99	8.4	ND
Water P-5	4/19/89	8.59	54	2.19	8.6	ND
Water P-3B	5/16/89	8.47	68	1.90	8.2	4
P-3B spiked with Se(IV)				4	3.8	4
				7	2.0	4
				12	1.3	4
				22	0.7	3
				102	0.1	3R
P-3B spiked with Se(VI)				4	3.8	4
				7	2.1	4
				12	1.4	3
				22	0.7	2
				102	0.1	1R

[a]Pond waters were collected from the following Central Valley ponds: MR=Martin Ranch, Tulare Co.; W=Ponds 3A and 3B Westfarmers, Kern Co.; P=Cells 1, 3 and 5 Peck Ranch, Fresno Co. (Fig. 1-1).

[b]Microbial numbers were evaluated by the density of colonies growing on R_2A medium streaked with pond water at the end of the experiment: 4=colonies extremely dense and confluent on all streaks; 3=colonies prolific and confluent on all but the 4th set of streaks; 2=no colonies on 4th set of streaks, few on 3rd set of streaks; 1=colonies sparsely distributed on 1st and 2nd streaks, no colonies on 3rd and 4th streaks; R=reduction of Se occurred and turned the pond water blood red.

ND, not determined

water containing 2,190 µg Se L^{-1} produced 7.5 times more DMSe than water containing 20 µg Se L^{-1}), the methylation efficiency decreased with increasing Se concentration. Selenium removal after 17 d of incubation was 100% at $\leq$20 µg Se L^{-1}, but at concentrations of 200 to 300 µg Se L^{-1}, removal was approximately 20% and at concentrations of above 1,900 µg Se L^{-1}, only 8.4% of the total Se inventory was removed. In general, as the EC increased, the Se concentration increased but the microbial methylation efficiency decreased. As pH increased, the microbial methylation efficiency increased logarithmically but there was little relationship between pH and either Se concentration or EC. Increasing the concentration of inorganic Se species, Se(IV) and Se(VI), in the pond water affected the overall percentage of Se inventory removed (Table 7). For every doubling in Se concentration, the efficiency of Se methylation decreased by approximately 50%. The percentage of Se removal was 8, 4, 2, 1, 0.7 and 0.1%, respectively, for Se concentrations of 2, 4, 7, 12, 22, and 102 mg Se L^{-1}. There was essentially no difference in the amount of DMSe produced when either Se(IV) or Se(VI) was added as the Se substrate. An assessment of microbial numbers indicated that Se(IV) was less toxic to aquatic bacteria than Se(VI). A decline in microbial numbers was observed at a lower concentration of Se(VI) (12 mg Se L^{-1}) than Se(IV) (22 mg Se L^{-1}). At the highest concentration tested, 102 mg Se L^{-1}, visible reduction of the two oxidized Se species to elemental Se occurred as the pond water turned blood red in color.

Reduction in salinity and selenium concentration

Dilution of the pond water caused a decrease in salinity and Se concentration. As the pond water salinity and Se concentration decreased, methylation values also declined compared with the undiluted controls (Fig. 2-1).

Aeration and agitation

Selenium methylation was optimal in shaken protein-amended pond water (Table 8). The shaken protein-amended pond water, incubated in a nitrogen atmosphere, only produced 76% of the DMSe produced by aerated, protein-amended water. Incubation of protein-amended pond water without shaking under air and nitrogen produced only 47% and 22%, respectively, of the activity of the optimum air-incubated protein-amended, shaken pond water. Levels of methylation in unamended water were very low. In general, water produced a greater than 2-fold increase in DMSe detected compared with statically-incubated water. A factor of 1000X separated the best treatment, shaken, casein-amended water incubated under air from the least effective, static pond water incubated under a nitrogen atmosphere.

Attached growth surfaces

The presence of additional microbial attachment sites increased methylation in unamended pond water over suspended microbial growth. Stimulation by these substances occurred in the following order: glass beads (3-mm) > ring lace > glass beads (0.45-0.52-mm) > unamended water (Table 9). Glass beads (3-mm) and ring lace more than doubled DMSe evolution compared with the unamended control. With the addition

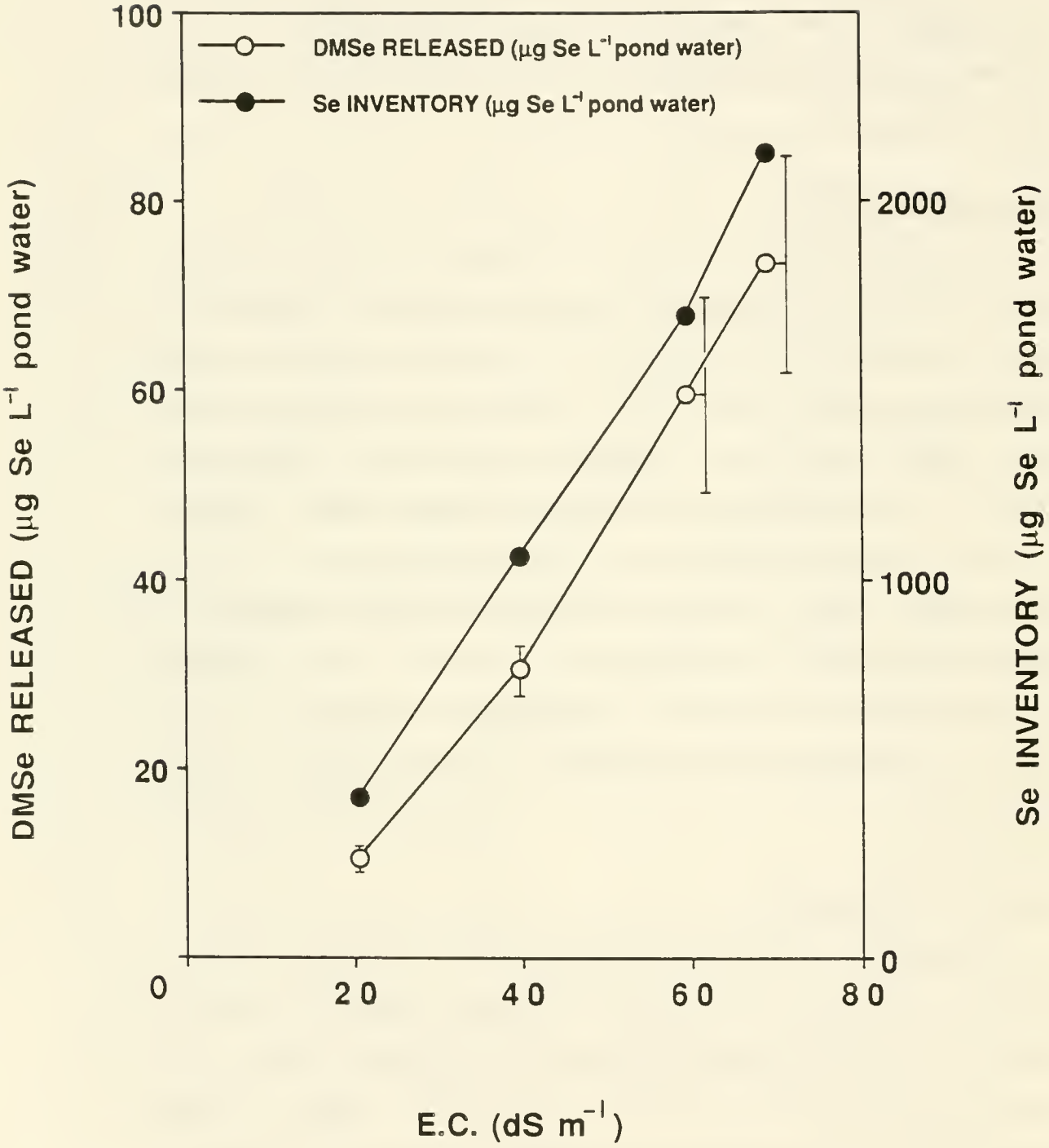

Fig. 2-1. The influence of pond water salinity and Se concentration on casein-mediated biomethylation.

Table 8. Influence of aeration and agitation on DMSe production.

Organic amendment	Incubation	Headspace atmosphere	Average DMSe emission (μg Se L^{-1} pond water)[a]
Untreated	Static	Air	0.5 ± 0.2a[b]
		Nitrogen	0.3 ± 0.1a
	Shaken	Air	1.2 ± 0.3b
		Nitrogen	1.9 ± 0.3b
Casein-amended	Static	Air	109.9 ± 4.7c
		Nitrogen	70.1 ± 7.9c
	Shaken	Air	314.0 ± 10.7d
		Nitrogen	235.6 ± 54.1d

[a]Mean values are given with standard errors (n = 5).

[b]Values followed by the same letter are not significantly different from each other (Duncan's new multiple range test, $P < 0.05$).

of peptone, there was no evidence of increased methylation in the presence of these physical materials.

Nitrates and nitrites

Both NO_3^- and NO_2^- ions (0.1 and 1 M) inhibited protein-stimulated methylation (Fig. 2-2). The NO_2^- ion was more inhibitory than NO_3^- at 0.1 M causing a 58% reduction in Se methylation compared with a 43% reduction by the NO_3^- ion after 18 days of incubation.

Sulfates

The addition of 0.1 and 1 M SO_4^{2-} to the pond water slightly increased (10%) Se biomethylation (Fig. 2-3) in peptone-amended pond water.

Alcohols

Methanol did not stimulate or inhibit DMSe emission from pond water at any of the concentrations tested.

Oils

None of the oils tested had any significant effect on Se methylation.

DISCUSSION

Investigations with seleniferous pond water using microscopy techniques, antibiotics, spread plate counts and culturing techniques indicate that bacteria are the principal Se methylating microflora in water. Previous work has shown that while most isolates obtained

Table 9. Influence of microbial attachment sites on biomethylation of Se.

Treatment	Physical materials	Average DMSe emission (μg Se L^{-1} pond water)[a]
Untreated	None	1.6 ± 0.1
	Large glass beads (3-mm)	5.1 ± 0.8
	Small glass beads (0.45-0.52-mm)	3.2 ± 0.9
	Ring lace (10 cm)	3.6 ± 0.4
	LSD	2.1
Casein	None	141.8 ± 23.1
	Large glass beads (3-mm)	145.3 ± 16.7
	Small glass beads (0.45-0.52-mm)	83.6 ± 17.5
	Ring lace (10 cm)	132.5 ± 19.0
	Polyurethane	99.1 ± 20.4
	LSD	65.2

[a]Mean values are given with standard errors (n=5, LSD, $P < 0.05$).

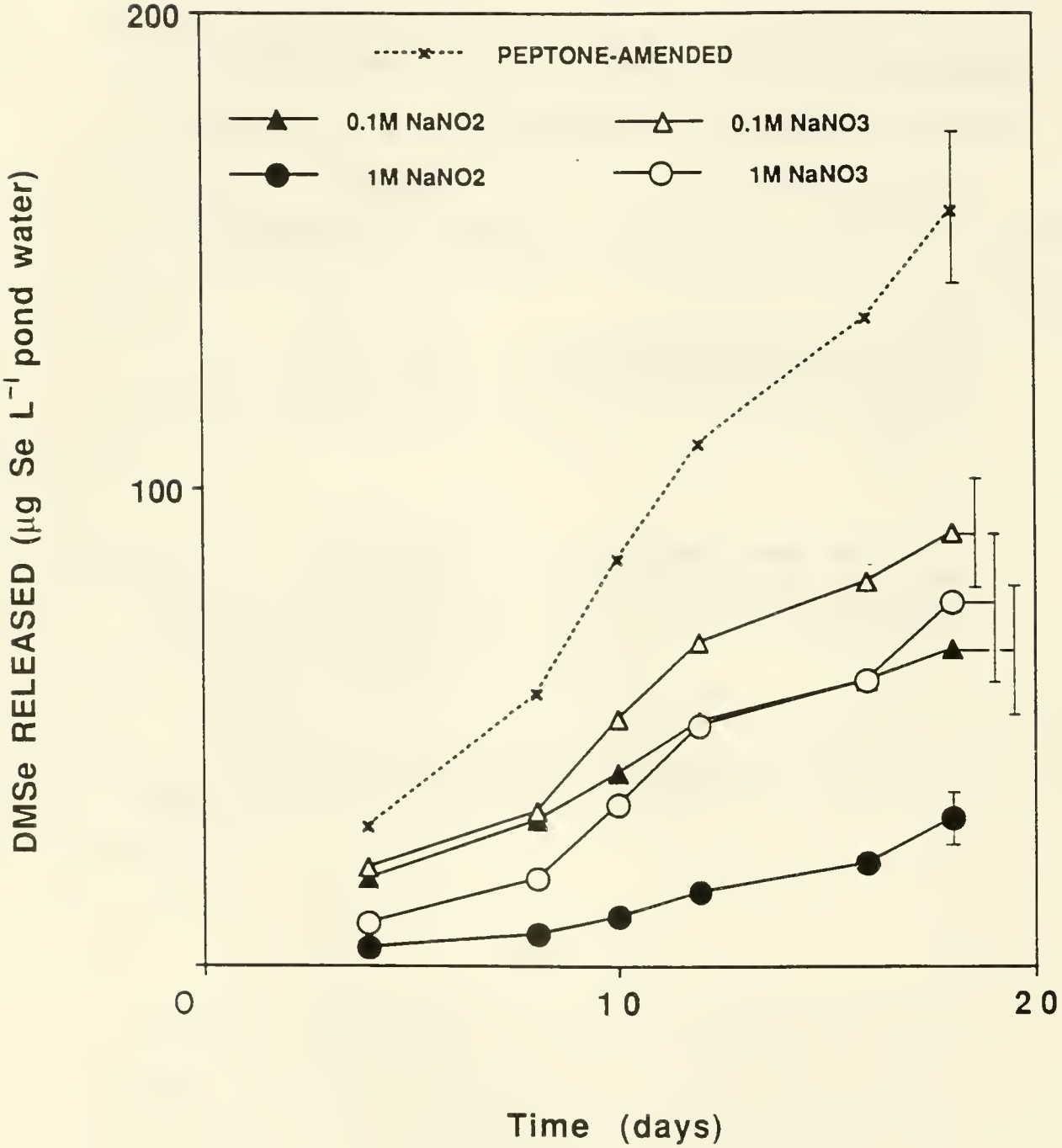

Fig. 2-2. Inhibition of peptone-mediated DMSe production by nitrate and nitrite ions.

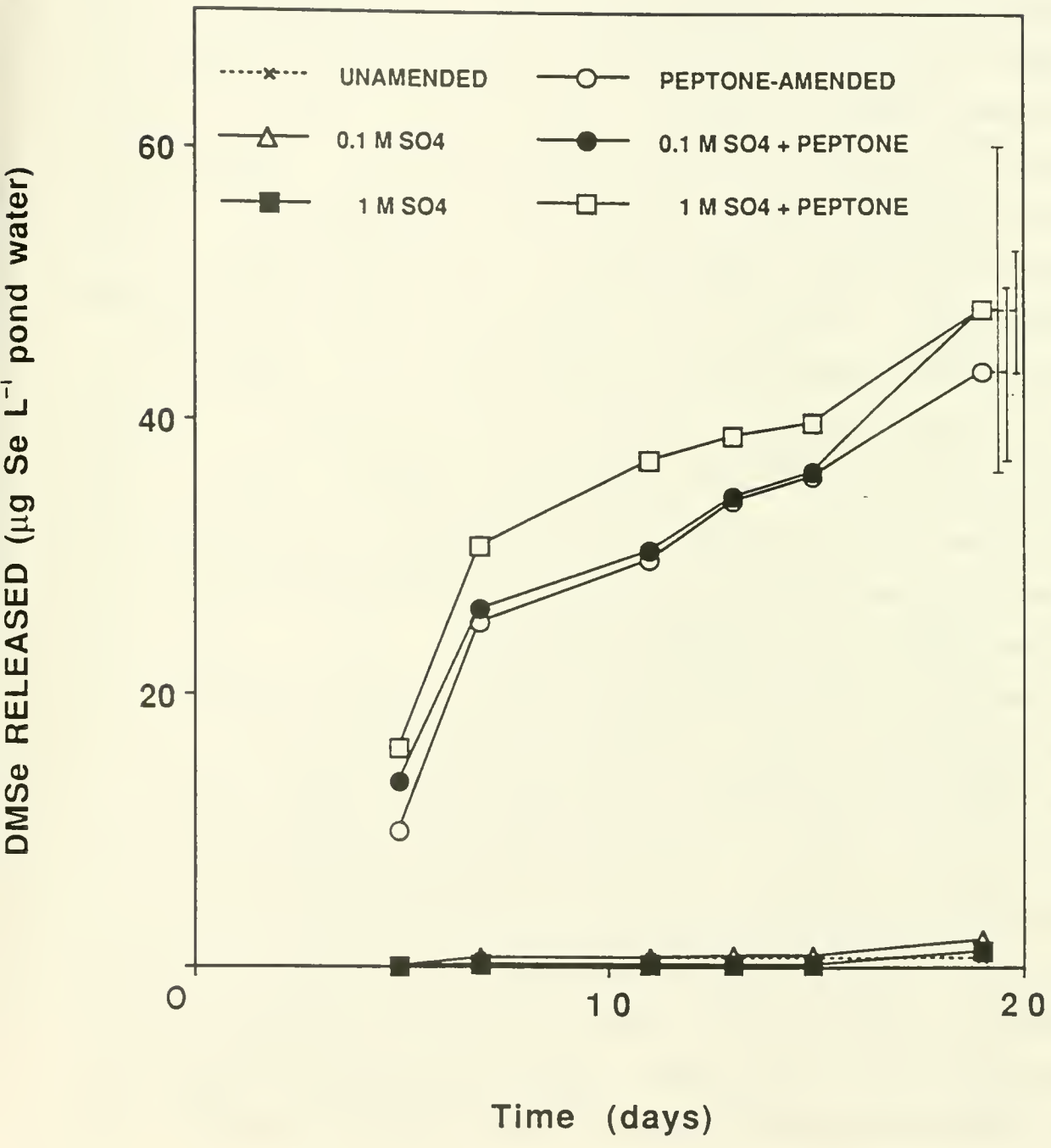

Fig. 2-3. Influence of sulfates on DMSe production in pond water.

from pond water were bacteria, the most active Se-methylating isolates in artificial media were fungi. Experiments with pure isolates of Alternaria alternata showed it to be an extremely efficient methylator of Se when cultured in a rich malt extract medium (Thompson-Eagle et al., 1989). However, subsequent studies indicated that when the fungus was incubated in pond water together with a simple carbon source (glucose), the stimulation in methylation was extremely poor (Thompson-Eagle and Frankenberger, 1990). Previous work has shown that fungi contribute little to the microbial activity in lake sediments (Saxena and Howard, 1977). The habitat of microorganisms is of great importance when studying environmental metalloid transformations. For instance, the ratio of fungi to bacteria in soils is often governed by the moisture content. Dry sites have higher fungal populations, while water-saturated environments favor bacteria (Hasebe et al., 1984). The major methylating microflora in dry, alkaline, seleniferous soils appear to be fungi (Karlson and Frankenberger, 1989).

Microbial nutrition and growth were found to be directly related to the Se methylation rates. The addition of casein to pond water caused a 1000-fold increase in microbial numbers and a 25-fold increase in DMSe emission. Bisogni and Lawrence (1973) also demonstrated that a doubling in net growth rates led to a 3- and 2-fold increase in the net mercury methylation under anaerobic and aerobic conditions, respectively. Demethylation and subsequent oxidation of disodium methanearsonic acid to carbon dioxide is also directly affected by the amount of organic substrates available to sustain microbial activity (Dickens and Hiltbold, 1967).

The consortium of Se-methylating bacteria was resistant to a number of antibiotics tested. Many of these antimicrobial agents reduced specific microbial populations without decreasing DMSe production. The application of bactericides and fungicides to the pond water amended with protein confirmed our hypothesis that bacteria are the principal methylators in alkaline, saline waters. Selenium biomethylation in the water significantly decreased with the addition of Penicillin G and Polymyxin B sulfate which are active against Gram-positive and Gram-negative bacteria, respectively. As expected, the response to the fungicides ranged from no effect to a positive one.

Many enzymes require specific activators, nonprotein prosthetic groups, and coenzymes which accept or donate functional groups in order to function. The methylation of Se involves both a reduction and a methylation step (Challenger et al., 1954; Doran, 1982). Therefore, potential coenzymes that are likely to be involved in transmethylation include: SAM and its precursors/derivatives, homocysteine and methionine, and reducing agents including thiols such as glutathione. Surprisingly, of all the potential coenzymes, activators and prosthetic groups tested, only methionine, homocysteine and reduced glutathione enhanced the stimulation of pond water Se biomethylation. The lack of effect of SAM and methylcobalamine on unamended pond water and their inhibitory effects on protein-amended water was unexpected. These compounds are biological methyl donors in many reactions including the microbial methylation of inorganic Se in cell-free extracts (Doran, 1982). However, this study was conducted *in vivo* rather than *in vitro*.

Factors such as removal and utilization of these coenzymes by non-methylators, diversion into other metabolic pathways and destruction by bacterial enzymes may have prevented these donors from reaching the active cell sites.

The action of reduced glutathione and homocysteine appears to be at least partly abiotic since sterile water generated DMSe in the presence of these membrane filter-sterilized compounds. Despite their abiotic contribution to Se methylation, DMSe production was considerably greater in non-sterile conditions. Homocysteine has also been found to stimulate methylmercury formation by both the fungus, Neurospora (Landner, 1971) and various unidentified bacteria (Grant and Tindall, 1986). In contrast, Fleming and Alexander (1972) found that the growth and methylation of Se by Penicillium was influenced by methionine but not homocysteine or homocystine. Methionine, a precursor of SAM, appears to have a nutritional rather than a methyl donor role in this study because the stimulation in methylation observed in methionine-amended water disappeared in the presence of peptone and little methylation was observed under sterile conditions in the presence of methionine.

Weres et al. (1989) confirmed the results of our coenzyme experiments. They suggested that the reasons why thiols such as glutathione and homocysteine are effective in stimulating Se methylation is that they increase the solubility of Se. This reaction renders the element more available to the methylating microbes in pond water and hence stimulates volatilization.

Of particular interest is the fact that many of these methylation coenzymes have been found to reverse Se toxicity in animals. For instance, glutathione is a major cellular thiol (Ganther, 1986) and is involved in Se incorporation into proteins via selenotrisulfide linkages (Jiang et al., 1982). In the mammalian liver, glutathione is an absolute requirement for the detoxification and anaerobic reduction of selenite to DMSe. Homocystine and creatine give protection against mammalian selenosis (Olson et al., 1958). Methionine aids in Se detoxification through the formation of methylated derivatives which can then be exhaled in the breath or excreted in the urine of animals (Levander, 1972; Levander and Morris, 1970) and provides protection against inorganic but not organic species of Se (Diplock, 1976). It seems likely that Se biomethylation is as important in animal Se detoxification as it is in various microbial species.

Selenium biomethylation in evaporation pond water did not show a corresponding increase in methylation with an increase in Se substrate concentration. This was observed for pond water spiked with a range of Se concentrations between 4 and 102 mg L^{-1} as well as for pond waters naturally containing Se concentrations ranging between 0.02 and 2.2 mg L^{-1}. This confirms the findings of Fleming and Alexander (1972) who demonstrated that at 10 mg L^{-1} Se(IV), a Penicillium sp. converted 13-24% of the added Se to DMSe, while at 100 mg L^{-1}, less than 2% of the Se inventory was methylated. In contrast, under anaerobic conditions, methylmercury emission was found to increase as the substrate concentration increased (Bisogni and Lawrence, 1973). Studies with aerobic mercury systems in lake sediments have shown that the production

of methylmercury increases with increasing inorganic mercury dosage up to 100 mg kg^{-1} sediment (Jensen and Jernelov, 1969). However, any further increase in concentration caused a sharp decrease in the methylmercury released. Jensen and Jernelov (1969) concluded that this was due to the substrate toxicity of the mercury methylating microorganisms. Selenium methylating organisms do not appear to be inhibited at high concentrations of Se since methylation continued even at the high Se levels. There was no upper or lower threshold for Se biomethylation between 17 µg L^{-1} and 102 mg L^{-1}. However, at 100 mg L^{-1} Se(IV) or Se(VI), the aquatic microorganisms also reduced Se into the elemental form which caused the water to become blood red in color. Selenium reduction to elemental Se is an alternative resistance mechanism utilized by many bacteria and fungi which enables survival in a seleniferous environment. The inorganic species Se(IV) and Se(VI) were equally effective substrates for methylation and reduction.

High Se concentrations in evaporation pond water are generally accompanied by high EC levels (Table 7). Salinization has been found to inhibit Se volatilization in soils (Karlson and Frankenberger, 1990), but dilution of the saline evaporation pond water decreased the efficiency of protein-mediated Se methylation despite the decrease in Se inventory which by itself would be expected to increase the water methylation capacity. The results of these experiments indicate that Se concentration, microbial methylating populations and nutrients are more critical limitations to Se methylation than either pH or high salinity. The methylating bacteria are alkalinophilic and well adapted to their harsh environment.

In addition to the presence of common ions such as sodium, sulfate and chloride in virtually molar concentrations, the pond water is high in trace elements such as arsenic, boron, chromium, lead, molybdenum, Se, silver, and uranium. The indigenous population of methylating microflora are extremely resistant to high levels of trace elements (Huysmans and Frankenberger, 1989). While high salt concentrations may limit the number of bacteria in evaporation pond water, these elements do not necessarily affect methylating microorganisms. In some cases, the presence of other ions can actually stimulate methylation. Karlson and Frankenberger (1988) reported that the addition of cobalt, zinc, and nickel to soil increased Se volatilization rates up to 2.5-fold. Fleming and Alexander (1972) found that Se(IV) alkylation increased with increasing SO_4^{2-} concentrations. The addition of 1 M SO_4^{2-} to peptone-amended water in this study caused a 10% increase in biomethylation.

Elevated concentrations of NO_2^- are known to be toxic to many bacteria (Focht, 1982). Both NO_2^- and NO_3^- inhibit anaerobic Se transformations (Oremland et al., 1989). Pond water methylation of Se was inhibited by both NO_3^- and NO_2^- at concentrations of 100 mM and above. Although studies by Cox and Alexander (1973, 1974) showed that NO_3^- did not inhibit either Se or As methylation by Candida humicola, the maximal concentration tested was an order of magnitude lower than the concentrations used in this study.

Optimum Se methylation in evaporation pond water occurs under aerobic conditions in well-mixed, protein-amended systems. Selenium methylation was also found to occur at lower redox levels in an anaerobic (under nitrogen), static system. Similarly, anaerobic

conditions were not a requirement for the reduction and methylation of arsenite in nutrient-supplemented pond water (Braman, 1975). However, lead methylation in lake water sediments amended with nutrient broth and glucose was found to be optimal under anaerobic conditions (Wong et al., 1975).

Microcolony growth is thought to protect organisms from the effects of chemicals, surfactants and biological predators (Costerson and Geesey, 1979). In addition, colonized surfaces provide a nutritional advantage as populations become more numerous (Costerson and Cheng, 1982). As a result of these factors, adherent, sessile bacteria outnumber planktonic organisms in most aquatic systems (Geesey et al., 1978). Many of the degradative processes that are so useful to man are carried out by aggregates of more than one species of adherent bacteria (Costerson and Cheng, 1982). Sewage treatment systems have been greatly improved by the inclusion of colonization surfaces at a rate of 20 m^2 per m^3 reactor volume (Weber, 1984). Selenium methylation rates from unamended evaporation pond water increased with the addition of inert substances such as glass beads, sand and nylon fibers. However, the same phenomenon was not observed when peptone was added to the water. This result was unexpected since peptone is a soluble mixture of peptides rather than an insoluble protein and therefore it should have no effect on the number of colonization sites available for the establishment of these microbes. However, the addition of this soluble mixture of peptides to the supersaturated saline solution could have caused the peptides to precipitate into particulate, colloidal

materials which could form colonizable surfaces for the microbes. It is also possible that with the added nutrients (peptone) available for microbial utilization, these organisms were able to multiply and form mini-aggregates which allowed for efficient Se metabolism so that any further addition of colonizable surfaces would have no further effect on methylation. Aggregates were observed to form in flasks during incubation with organic amendments and when examined microscopically, they were found to consist of large particles enmeshed among hundreds of different bacterial cells and salt granules.

Methanol is a commonly used carbon source in the wastewater industry (James Montgomery Consulting Engineers, Inc., 1985) where it is employed for the enrichment of denitrifying bacteria. Methanol was found to have little effect on Se methylation in evaporation pond water. Linseed oil has been reported to be protective against Se poisoning in mammals (Diplock, 1976). It is believed that linseed oil changes the Se into a less toxic chemical form. None of the animal or vegetable oils tested in pond water had any effect on microbial Se methylation. Enhancement of Se methylation is apparently specific to proteins and protein-containing compounds. These findings are consistent with previous work indicating that Se methylating microorganisms are not stimulated by organic amendments such as mono-, poly- and acidic saccharides (Thompson-Eagle and Frankenberger, 1990, Chapter 1).

CONCLUSION

Transmethylation of Se can be optimized through the enhancement of growth and metabolism of a consortium of indigenous alkalinophilic methyl-ating microorganisms. These studies indicate that biomethylation can be promoted in a well-mixed, protein and cofactor-amended system. These microorganisms are resistant to a large number of toxic trace elements naturally present in pond water. The increasing salinity of the pond waters during the summer months should not cause a major reduction in the methylation efficiency. Any inhibition occurring as a result of increasing salinity is likely to be at least partially offset by the promotion of Se methylation with increasing temperature (Thompson-Eagle and Frankenberger, 1990). With these parameters in mind, it should be possible to design a pilot bioreactor and test its ability to deselenify hazardous agricultural drainage water and/or industrial effluents. This bioreactor may be a feasible method to reduce the Se load in areas such as the San Joaquin Valley in California.

FUTURE RESEARCH

High selenium (Se) levels in evaporated agricultural drainage water continue to be a problem for both indigenous and migrating wildlife. The use of biological dissipation to remove Se from contaminated water sources including agricultural drainage water is a technology that is in its infancy. Over the last two years we have managed to characterize and dramatically accelerate the reaction whereby aquatic microbes remove toxic Se species from the water and liberate it into its non-hazardous, volatile form, dimethylselenide (DMSe). The time is now ripe to apply these technologies into a practical deselenification water process which beleagured growers will be able to use in situ. According to the U.C. Salinity/Drainage Task Force panel (1989), there are no other preventative methods available and deselenification may be a novel but practical method to combat the yearly increase in selenium concentrations in the ponds. Each grower has to donate roughly 10% of his land to an evaporation pond facility in order to continue farming in certain areas of the San Joaquin Valley. Without funding, no research will occur on water deselenification.

Future work should concentrate on successfully developing a deselenification water treatment process using the information collected from our laboratory and field studies. This work would involve testing a number of different engineering methodologies in combination with previously researched optimum variables. Different types of deselenification bioreactors need to be assessed including activated sludge

systems, aerobic lagoons, trickling filter, rotating biological contactors, and high-rate oxidation ponds. The drainage water would be introduced into the bioreactor in the presence of established aerobic bacterial cultures which would serve as seed. An aerobic environment could be maintained by use of diffused or mechanical aeration. Various protein amendments, cofactors and vitamins would be added to promote volatilization. Sources of economical nutrients necessary for fuelling the reaction would continue to be screened. Each reactor would need to be characterized in terms of kinetics of Se removal, optimal protein loads, biochemical oxygen demand, residence time and influent and effluent Se concentration, and need for a secondary oxidation treatment. If successful, the most efficient reactor could be scaled up into a much larger pilot study in the field. It should be possible to make an estimate of the cost per acre-foot of an agricultural drainage water treatment system.

Laboratory studies on the microorganisms that methylate Se in the evaporation pond water would also be of interest. Characterization of these bacterial consortia with emphasis on the genetic implications of Se biomethylation would allow Se-contaminated sites to be evaluated for their methylation capabilities and hence deselenification potential.

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